

**Investigating the Cardiovascular Effects of  
Antiretroviral Drugs In a Lean and High Fat/Sucrose  
Diet Rat Model of Obesity: An *in vivo* and *ex vivo*  
Approach**

by

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degree of Master of Science in Medical Sciences in the Faculty  
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## **DECLARATION:**

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## Abstract

### **Introduction:**

An interaction exists between cardiovascular risk factors (e.g. obesity) and antiretroviral treatment (ART) in the pathogenesis of cardiovascular disease. While ART reverses HIV-related weight loss, studies investigating ART effects in the context of obesity are lacking.

### **Objective:**

To investigate the effects of Odumine® (first-line fixed ART-drug combination) on several cardio-metabolic parameters in a high fat/sucrose diet (HFD) rat model of obesity.

### **Methods:**

**Groups:** Lean, untreated (C/-ART); HFD, untreated (HFD/-ART); Lean, treated (C/+ART); HFD, treated (HF/+ART). **Sample size:** n = 28 - 34 / group; male Wistar rats. The HFD feeding programme followed for 16 weeks and ART treatment programme for the last 6 weeks of HFD feeding programme. **The Endpoints** measured included: Food and water consumption for the first 31 days during the drug treatment programme; Biometric measurements: Total body mass (TBM), intra-peritoneal (IP) fat mass, heart mass and liver mass; Blood and serum: Glucose, insulin, total cholesterol (TC), triglycerides (TGs); Thiobarbituric acid reactive substance (TBARS) and conjugated dienes (CD); Isolated heart perfusion: Functional recovery (Global ischaemia-reperfusion) and infarct sizes (Regional ischaemia-reperfusion); Western blot (Pre- and post-ischaemia-reperfusion): Nitric oxide synthase (NOS) signalling (eNOS, PKB/Akt and AMPK), indicators of reactive oxygen species (ROS) (Nitrotyrosine and p22 phox) and an indicator of pro-inflammatory NF-κB signalling (IκBα) in heart tissue.

### **Results:**

The **HFD** was validated by increases in TBM, IP fat mass and heart mass. The HFD was further validated by increased TG and TBARS levels (lipid peroxidation). Pre-ischaemia-

reperfusion, the HFD was associated with reduced oxidative stress (nitrotyrosine and p22 phox) vs. C/-ART. Reduced oxidative stress was associated with increased activation of the pro-inflammatory NF- $\kappa$ B pathway. The HFD upregulated eNOS post-ischaemia-reperfusion, downregulated PKB/Akt and increased NF- $\kappa$ B activation. Lastly, the HFD was associated with reduced functional recovery vs. C/-ART and HF/+ART, and increased infarct size vs. C/-ART and HF/+ART.

**ART** treatment did not affect the food and water consumption, was associated with reduced insulin levels vs. C/-ART and HF/+ART, increased TC levels vs. C/-ART and elevated levels of oxidative stress (increased TBARS) vs. C/-ART. Pre-ischaemia-reperfusion, ART improved oxidative stress in heart tissue (reduced p22 phox) vs. C/-ART and reduced inflammation (downregulated I $\kappa$ B $\alpha$ ). Post-ichaemia-reperfusion, ART upregulated eNOS in the heart tissue, downregulated PKB/Akt and increased oxidative stress (nitrotyrosine) vs. C/-ART. ART *per se* did not affect functional recovery or infarct size.

**The HFD combined with ART** increased liver mass vs. HF/-ART. Pre-ischaemia-reperfusion, HFD/+ART improved oxidative stress (decreased nitrotyrosine and p22 phox) vs. HF/-ART, but decreased NF- $\kappa$ B activation vs. HF/-ART. Post-ischaemia-reperfusion, ART combined with HFD upregulated eNOS, downregulated PKB/Akt and increased oxidative stress (p22 phox) vs. HF/-ART and C/+ART. Post-ischaemia-reperfusion, ART with HFD seemed to improved functional recovery vs. HF/-ART and ameliorated the increased infarct size vs. HF/-ART.

### **Discussion and Conclusion:**

Our study demonstrates the detrimental effect of HFD/obesity on cardiovascular health. Interestingly, when combined with ART, cardiovascular function seem to be improved vs. HFD/-ART. ART alone, affected cardiovascular health to a lesser extent in our study vs. HF/-ART and HF/+ART, and did not affect functional recovery and infarct size vs. C/-ART at all.

## Opsomming

### Inleiding:

'n Interaksie bestaan tussen kardiovaskulêre risikofaktore (bv. vetsug) en antiretrovirale behandeling (ART) in die ontwikkeling van kardiovaskulêre siekte. Terwyl ART HIV-verwante gewigsverlies kan omkeer, is daar min bekend oor ART-effekte in die konteks van vetsug.

### Doelstelling:

Om die effekte van Odumine® (eerste-linie ART-middel kombinasie) op verskeie kardio-metaboliese parameters in 'n hoë vet/sukrose dieet (HFD) rotmodel van vetsug te bepaal.

### Metodes:

**Groepe:** Kontrole/geen ART (C/-ART); HFD/geen ART (HFD/-ART); Kontrole/met ART (C/+ART); HFD/met ART (HFD/+ART). Aantal proefdiere: n = 28-34/groep; manlike Wistar rotte. Die HFD voedingsprogram was 16 weke lank en ART was gedurende die laaste 6 weke van die voedingsprogram daaglik toegedien.

**Die Eindpunte was:** Voedsel- en waterinname vir die eerste 31 dae van die middeltoedieningsprogram; Biometriese metings: Totale liggaamsmassa (TLM), intra-peritoneale (IP) vetmassa, hart- en lewermassa; Bloed and serum: Glukose, insulien, totale cholesterol (TC), triglisserieerde (TGs); "Thiobarbituric acid reactive substance" (TBARS) and gekonjugeerde diëne (CD); Geisoleerde hartperfusies: Funksionele herstel (Globale isgemie-herperfusie) and infarkgrootte (Regionale isgemie-herperfusie); Western blot (Voor- na na-isgemie-herperfusie): Stikstofoksied sintase (NOS) seintransduksie (eNOS, PKB/Akt en AMPK), merkers van reaktiewe suurstofspesies (ROS) (Nitrotirosien en p22 phox) en 'n merker van pro-inflammatoriese NF- $\kappa$ B seintransduksie (I $\kappa$ B $\alpha$ ).

### Resultate:

Die HFD is bevestig deur 'n betekenisvolle toename in die TLM, IP vetmassa en hartmassa. Die HFD was verder bevestig deur die toename in die TG en TBARS vlakke

(lipiedperoksidasie). Die HFD was geassosieer met verlaagde eindpunte van oksidatiewe stres in die hartweefsel (nitrotirosien and p22 phox) versus C/-ART. Verlaagde oksidatiewe stres was met verhoogde NF-  $\kappa$ B aktivering geassosieer. Isgemie-herperfusie het eNOS in die HFD hartweefsel opgereguleer, PKB/Akt afgereguleer en NF-  $\kappa$ B aktivering verhoog in die HFD groep. Laastens was die HFD was geassosieer met verlaagde funksionele herstel versus C/-ART en HF/+ART, en verhoogde infark grootte versus C/-ART en HF/+ART.

**ART toediening** het geen effek op die voedsel- en waterinname gehad nie, was geassosieer met verlaagde insulienvlakke versus C/-ART en HF/+ART, verhoogde TC vlakke versus C/-ART, en verhoogde oksidatiewe stres (verhoogde TBARS) versus C/-ART. Voor blootstelling aan isgemie-herperfusie, het ART-toediening oksidatiewe stres in hartweefsel verlaag (verlaagde p22 phox) versus C/-ART en pro-inflammatoriese NF-  $\kappa$ B aktivering verhoog (afgereguleerde I $\kappa$ B $\alpha$ ). Na blootstelling aan isgemie-herperfusie het ART-toediening eNOS opgereguleer, PKB/Akt afgeruguleer en oksidatiewe stres verhoog (nitrotirosien) versus C/-ART. ART het geen effek op funksionele herstel of infark grootte gehad nie.

**ART-toediening aan HFD diere** het die lewermassa verhoog versus HF/-ART. Voor isgemie-herperfusie blootstelling, het ART-toediening in HFD diere oksidatiewe stres verbeter (verlaagde nitrotirosien and p22 phox) versus HF/-ART, maar NF- $\kappa$ B aktivering verlaag versus HF/-ART. Na isgemie-herperfusie-blootstelling, het ART-toedining in HFD diere eNOS opgereguleer, PKB/Akt afgereguleer en oksidatiewe stres verhoog (p22 phox) versus HF/-ART en C/+ART. Verder was die funksionele herstel beter en die infark-grootte kleiner na isgemie-herperfusie in die HFD-groep.

### **Bespreking en Gevolgtrekking:**

Ons studie het die skadelike effekte van HFD/vetsug op kardiovaskulêre gesondheid gedemonstreer. 'n Interessante waarneming was dat die kombinasie van vetsug met ART tot 'n mate kardiovaskulêre funksie verbeter het versus HFD/-ART. ARV self het kardiovaskulêre gesondheid tot 'n mindere mate geaffekteer versus HF/-ART en HF/+ART, en het nie funksionele herstel en infarkt grootte in kontrole diere geaffekteer nie.

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## LIST OF ABBREVIATIONS

AHA	: American Heart Association
AMP	: Adenosine Monophosphate
AMPK	: 5' Adenosine Monophosphate-activated Protein Kinase
ANOVA	: Analysis of Variance
AO	: Aortic Output
APS	: Ammonium Persulfate
AR	: Area at Risk
ART	: Antiretroviral Therapy
BMI	: Body Mass Index
BSA	: Bovine Serum Albumin
CD-4	: Cluster of Differentiation-4
CF	: Coronary Flow
CO	: Cardiac Output
CVD	: Cardiovascular Disease
D:A:D	: Data Collection on Adverse Events of Anti-HIV Drugs
d. H <sub>2</sub> O	: Distilled Water
DNA	: Deoxyribonucleic Acid
dP/dT <sub>max</sub>	: Maximum Rate of the Left Ventricular Pressure Rise
dP/dT <sub>min</sub>	: Maximum Rate of the Left Ventricular Pressure Fall
EC	: Endothelial Cell
ECL	: Enhanced Chemiluminescence
ED	: Endothelial Dysfunction
EDTA	: Ethylenediaminetetraacetic Acid
EFV	: Efavirenz
ELISA	: Enzyme-Linked Immunosorbent Assay
eNOS	: Endothelial Nitric Oxide Synthase
FDC	: Fixed Drug Combination
FTC	: Emtricitabine
GBD	: Global Burden of Disease
HAART	: Highly Active Antiretroviral Therapy
HDL	: High Density Lipoprotein

HFD	:	High Fat/Sucrose Diet
HI	:	Human Immunodeficiency
HIV/AIDS	:	Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome
HR	:	Heart Rate
IgG	:	Immunoglobulin Gene
IHD	:	Ischaemic Heart Disease
I $\kappa$ B $\alpha$	:	Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B-cells Inhibitor, Alpha
MI	:	Myocardial Infarction
mRNA	:	Messenger RNA
N	:	Sample Size
NCD	:	Non-Communicable Disease
NNRTI	:	Non-NRTI
NO	:	Nitric Oxide
NOS	:	Nitric Oxide Synthase
NRTI	:	Nucleoside/Nucleotide Reverse Transcriptase Inhibitors
PI	:	Protease Inhibitors
PKB/Akt	:	Protein Kinase B
PLWH	:	People Living with HIV/AIDS
PMSF	:	Phenylmethylsulfonyl Fluoride
PSP	:	Peak Systolic Pressure
RNA	:	Ribonucleic Acid
ROS	:	Reactive Oxygen Species
RPM	:	Revolutions per Minute
SA	:	South Africa
SABS	:	South African Bureau of Standards
SANS	:	South African National Standards Document
SDS	:	Sodium dodecyl sulfate
SDS-PAGE	:	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SEM	:	Standard Error of the Mean
SSA	:	Sub-Saharan Africa

SV	:	Stroke Volume
TBARS	:	Thiobarbuturic Acid Reactive Substance
TBM	:	Total Body Mass
TC	:	Total Cholesterol
TDF	:	Tenofovir Disoproxil Fumarate
TEMED	:	1,2-Bis(dimethylamino)ethane
TG	:	Triglyceride
TTC	:	2,3,5-Triphenyltetrazolium Chloride
UCT	:	University of Cape Town
US	:	University of Stellenbosch
USA	:	United States of America
VA	:	Viable Area
WHO	:	World Health Organisation
Wk	:	Kinetic Power
Wp	:	Pressure Power
Wt	:	Total Power



## UNITS OF MEASUREMENT

%	:	Percentage
°C	:	Degree Celsius
g	:	Gram
kDa	:	Kilo Dalton
kg	:	Kilogram
L	:	Litre
m	:	Meter
mg	:	Milligram
min	:	Minute
ml	:	millilitre
mmHg	:	Pressure
mmol	:	Milimol
mol	:	Molar
mW	:	Miliwatt
ng	:	Nanogram
nM	:	Nanomolar
v	:	Volume
μ	:	Micro
μl	:	Microliter
μmol	:	Micromolar

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## Chapter 1 - Literature Review

### 1.1. General Introduction to Study

According to the World Health Organisation (WHO), the burden of disease in high-income countries consists predominantly of non-communicable diseases (NCDs) such as cardiovascular disease (CVD), while low- and middle-income countries are experiencing a double burden of an increasing NCD prevalence as well as an on-going communicable (infectious) disease epidemic (Alwan, 2010; Mathers *et al.*, 2008; Remais *et al.*, 2012). CVD remains the biggest contributor to the global burden of disease (GBD) in terms of mortality and premature mortality, while human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) ranks 6<sup>th</sup> globally and number one in Sub-Saharan Africa (SSA) (Alwan, 2010; Lozano *et al.*, 2013; Byass *et al.*, 2013). Projections further indicate that both CVD and the number of people living with HIV/AIDS (PLWH) are currently on the rise (especially in the developing world) and often affect the same communities (Alwan, 2010; Remais *et al.*, 2012; Lozano *et al.*, 2013; Mathers and Loncar, 2006).

Before the introduction of antiretroviral therapy (ART), HIV/AIDS was a fatal disease, characterised by severe weight loss and the occurrence of opportunistic infections, but ART revolutionised HIV/AIDS care, transforming it into a chronic, but manageable, disease requiring lifelong treatment (Sharp and Hahn, 2011; De Cock *et al.*, 2012; Ruelas and Greene, 2013). Soon after the introduction of ART, reports on metabolic abnormalities (such as insulin resistance and hypercholesterolaemia) and anthropometric abnormalities including lipodystrophy (lipohypertrophy in the abdominal area and/or lipoatrophy in the peripheral area) associated with ART-use started to emerge (Levitt *et al.*, 2011; Mayosi *et al.*, 2009).

Furthermore, traditional cardiovascular risk factors such as overweight and obesity seen in the general population have become more prevalent in HIV/AIDS population (Remais *et al.*, 2012; Mayosi *et al.*, 2009; Islam *et al.*, 2012; Fedele *et al.*, 2011). Higher rates of CVD have previously been observed in HIV/AIDS populations compared to the general population (Remais *et al.*, 2012; Islam *et al.*, 2012; Fedele *et al.*, 2011). The rising

prevalence of CVD in HIV/AIDS populations is of particular concern in regions such as SSA and developing countries including South Africa (SA) that are currently undergoing epidemiological transition against the background of very high HIV/AIDS rates (Mathers and Loncar, 2006; WHO, 2010; Narayan *et al.*, 2014). The increasing numbers of PLWH are as a result of the success of ART (improved life expectancy and health in general) and the dramatic up-scaling of HIV/AIDS treatment roll-out programmes (Mathers and Loncar, 2006; WHO, 2010; Narayan *et al.*, 2014).

Epidemiological transition in lower-income countries is resulting in the convergence of NCDs such as CVD and still highly prevalent communicable diseases such as HIV/AIDS (Remais *et al.*, 2012; Signorini *et al.*, 2012; Fedele *et al.*, 2011). Furthermore, CVD is emerging as a major contributor in terms of both mortality and morbidity in PLWH (Remais *et al.*, 2012; Signorini *et al.*, 2012; Fedele *et al.*, 2011), while the relationship between HIV/AIDS and CVD (and their risk factor profiles) is not yet fully understood. (Remais *et al.*, 2012; Signorini *et al.*, 2012; Fedele *et al.*, 2011). Factors, such as the increased incidence of CVD in PLWH and the fact that the mechanistic interplay between CVD, HIV/AIDS and risk factor profiles of these diseases is not yet understood, warrant the need for further scientific investigation.

## **1.2. Cardiovascular Disease (CVD)**

### **1.2.1. Ischaemic Heart Disease (IHD): Overview**

Ischaemic heart disease (IHD), or coronary artery disease, is the greatest contributor to CVD mortality globally. Atherosclerosis plays a central role in the development of IHD (American Heart Association (AHA); Reviewed: July, 2015; Naseem, 2005; Chabra N, 2009). The pathogenesis of atherosclerosis involves arterial plaque-formation due to a high cholesterol-rich fraction in the blood (AHA; Reviewed: July, 2015; Naseem, 2005). The continuous lipid deposition of this cholesterol-rich fraction in the arterial wall progressively leads to a decreased arterial lumen diameter and restricts arterial blood flow (AHA; Reviewed: July, 2015; Naseem, 2005). This reduced blood supply (also oxygen supply) to an area of tissue results in ischaemia (AHA; Reviewed: July, 2015; Naseem, 2005).

More specifically, the aetiology of atherosclerosis starts with the passive low-density lipoprotein (LDL) -diffusion into the arterial wall where it becomes trapped (due to its association with protein moiety, apolipoprotein B<sub>100</sub> matrix proteoglycans) (Naseem, 2005; Barbaro, 2003). LDLs then oxidatively changed through exposure to reactive oxygen species (ROS) and reactive nitrogen species (produced by monocytes and macrophages) in a protective response to localised damage caused by the LDL (Naseem, 2005; Barbaro, 2003). Oxidised LDL then furthermore leads a pro-inflammatory response in the surrounding cells (the production of chemokines such as monocytes, chemotactic protein-1 and growth factors) (Naseem, 2005; Barbaro, 2003). Oxidised LDL also stimulates the expression of adhesion molecules such as P-selectin, vascular adhesion molecule-1 and intercellular adhesion molecule-2 in the endothelial cells (ECs) (Naseem, 2005; Barbaro, 2003). The increased amount of adhesion molecules on the EC's surface causes entry of recruited monocytes into the arterial wall (Naseem, 2005).

Ultimately, this process advances to the formation of lipid-laden foam cells (Naseem, 2005) and a necrotic core of oxidized lipids, which is highly thrombotic (Naseem, 2005). The highly thrombotic core of the plaque subsequently ruptures to form platelet-rich thrombi (Naseem, 2005).

### **1.2.2. Epidemiology of Cardiovascular Disease (CVD)**

CVD is the top cause of mortality in the world although it has substantially decreased in high-income countries over the last 20 years (due to population wide intervention strategies), while low- and middle-income countries have experienced a substantial increase in CVD mortality (Alwan, 2011; Mathers *et al.*, 2008; Malaza *et al.*, 2012). CVD mortality in low- and middle-income countries currently accounts for more than 80 % of the global CVD mortality rate (Alwan, 2011; Mathers *et al.*, 2008; Malaza *et al.*, 2012). In 2008, 39 % of NCD deaths (36 million) under the age of 70 were due to CVD, while in 2010, approximately 17 million deaths (48 % of all NCD deaths) were due to CVD (Alwan, 2011; WHO, 2011). More specifically, IHD remained the most prominent contributor to global mortality (almost 13 million; 13.3 % of total deaths globally) in 2010 (Alwan, 2011; Lozano *et al.*, 2013).

Evidence, in terms of mortality and morbidity rates, also suggests that CVD is evolving into a major public health concern in SSA (Dalal *et al.*, 2011; Tibazarwa *et al.*, 2009). The WHO projected a doubling of IHD rates in the SSA Region by 2030 (Adeboye *et al.*, 2012). In SA, CVD, and in particular IHD, is one of the increasing causes of NCD mortality in all population groups (Norman *et al.*, 2006; Levitt *et al.*, 2011; Tibazarwa *et al.*, 2009). “The Heart of Soweto” study found cardiovascular risk factors highly prevalent in an urban SA community: Obesity (43 %), systolic or diastolic hypertension (33 %), and elevated total cholesterol (TC) levels (13 %; non-fasting) (Tibazarwa *et al.*, 2009).

Recent global projections also indicate an increase of 6 million deaths (from approximately 16.7 million to about 23.9 million) due to CVD over the next two decades globally, while IHD is expected to remain one of the top three leading causes of the GBD (Alwan, 2011; Dalal *et al.*, 2011; Islam *et al.*, 2012).

### **1.2.3. Cardiovascular Risk Factors**

Traditional cardiovascular risk factors can be classified as controllable/modifiable or uncontrollable/non-modifiable (Fedele *et al.*, 2011; WHO, 2012). They affect physiological pathways and/or processes such as blood pressure control directly or indirectly and subsequently lead to the pathogenesis of, and eventual progression toward, CVD (Fedele *et al.*, 2011; WHO, 2012) (**Table 1.1**).

**Table 1.1.** *Modifiable and Non-Modifiable Cardiovascular Risk Factors.* **Abbreviations:** LDL: Low-Density Lipoprotein; HDL: High-Density Lipoproteins (Poulter, 2003).

Modifiable Risk Factors	Non-modifiable Risk Factors
<ul style="list-style-type: none"> <li>• High LDL cholesterol</li> <li>• High blood pressure</li> <li>• Smoking</li> <li>• Low HDL cholesterol</li> <li>• Lack of exercise</li> <li>• Diabetes and glucose intolerance</li> <li>• Left ventricular hypertrophy</li> <li>• Central obesity</li> </ul>	<ul style="list-style-type: none"> <li>• Age</li> <li>• Sex</li> <li>• Family history</li> <li>• Ethnic origin</li> <li>• Birth weight</li> </ul>

Total cardiovascular risk and the rate of progression toward CVD depend on the amount of cardiovascular risk factors present, as well as the degree of intensity that each risk factor presents itself (WHO, 2011). Intervention anywhere along the continuum of events that may lead to CVD could disrupt the pathophysiological process (Fedele *et al.*, 2011).

### 1.3. Overweight and Obesity

#### 1.3.1. Introduction

Overweight/obesity is recognised by the WHO as a chronic disease, characterised by an increase in total body mass (TBM) and adiposity due to an imbalance between energy intake and expenditure leading to multiple comorbidities such as hypertension, insulin resistance and diabetes (Haslam and James, 2005; Van der Merwe and Pepper, 2006). Overweight/obesity also negatively impacts cardiovascular health directly and/or indirectly and is thus classified as a major cardiovascular risk factor (WHO, 2014). The WHO furthermore describes obesity as “one of the most blatantly visible, yet most neglected, public health problems that threatens to overwhelm both more and less developed countries” and classifies a person as being overweight if the body mass index (BMI) falls

between 25-29.9 kg / m<sup>2</sup> and obese as having a BMI of greater than 30 kg / m<sup>2</sup> (Haslam, 2005; Lavie *et al.*, 2009; Tchernof and Després, 2013).

### **1.3.2. Epidemiology of Obesity**

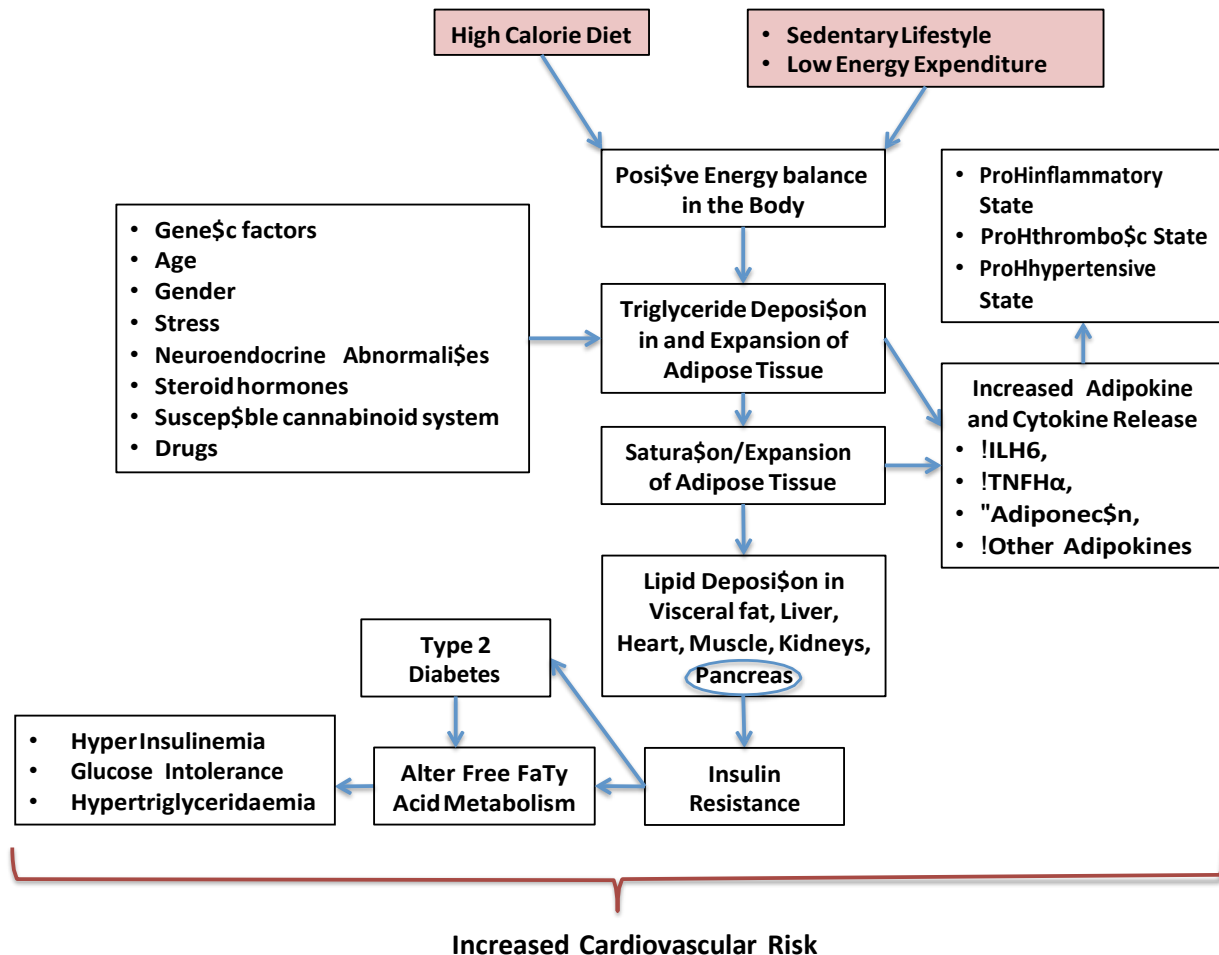
An estimated 205 million men and 297 million women over the age of 20 were obese in 2008, a total of more than half a billion adults worldwide (Alwan, 2011). Obesity is also the 6<sup>th</sup> most important risk factor contributing to the overall GBD worldwide (Haslam and James, 2005). It is estimated that more than 2.8 million people die each year as a result of being obese (5 % of global deaths) with women in particular significantly more obese than men (prevalence twice as high compared to that of men in some communities) (Alwan, 2011; Levitt *et al.*, 2011). Approximately 1.1 billion adults and 10 % of children are currently classified as overweight/obese (Haslam and James, 2005). More recently, in 2014, it was estimated by the WHO that 39 % of the world's population was obese (WHO, 2014).

Overweight/obesity among men and women is not only a great health challenge in high-income countries, but also a growing health concern in the lower-income countries and regions such as SSA (Alwan, 2011; Lim *et al.*, 2012; Dalal *et al.*, 2011; Prentice M, 2006). According to the WHO, 15 % - 24.9 % of SA's population older than 18 years of age is classified as obese compared to 39 % of the world's population (WHO, 2014). As is the case with developed countries, obesity is on the rise in SA (highest rate of obesity among SSA countries and is expected to take an even stronger hold as the coverage of SA's ART-programme increases and HIV/AIDS related weight loss is reversed (Malaza *et al.*, 2012; Strijdom, 2012; Mbanya *et al.*, 2014; Puoane *et al.*, 2002).

### **1.3.3. Overweight/Obesity and Comorbidities Associated With Cardiovascular Disease (CVD)**

Obesity-associated comorbidities include hypertension, type 2 diabetes mellitus, dyslipidaemia, certain cancers, and CVD (Alwan, 2011; Lavie *et al.*, 2009; Tchernof and Després, 2013). These obesity-related comorbidities can manifest through many complex physiological pathways (Alwan, 2011; Lavie *et al.*, 2009; Tchernof and Després, 2013).

Central adiposity in particular poses a great cardiovascular and health risk, as it is the driving force behind the development of most obesity-associated comorbidities such as insulin resistance and ectopic triglyceride (TG) deposition in the liver, heart, pancreas, and kidneys (**Fig. 1.1**) (Tchernof and Després, 2013).



**Fig. 1.1.** Obesity and Factors Associated with Increased Cardiovascular Risk.

**Abbreviations:** IL-6: Inter Leukin-6; TNF- $\alpha$ : Tumor Necrosis Factor Alpha. **Symbols:**  $\uparrow\uparrow$  = Increase;  $\downarrow\downarrow$  = Decrease. (Tchernof and Després, 2013; Alwan, 2011; Fedele *et al.*, 2011; Tchernof and Després, 2013; Bastien *et al.*, 2014; Mokdad *et al.*, 2003)

Dyslipidaemia is a major contributor to health complications in obesity and can be associated by any of the following: elevated TC, TG, LDL cholesterol, non-high density lipoprotein (HDL) cholesterol, apolipoprotein-B, and small dense LDL levels, or decreased HDL cholesterol levels (Alwan, 2011; Fedele *et al.*, 2011; Tchernof and Després, 2013).

## **1.4. Human Immunodeficiency Virus and Acquired Immunodeficiency Syndrome (HIV/AIDS)**

### **1.4.1. Introduction**

HIV/AIDS was first described by the Centres for Disease Control and Prevention in the United States of America (USA) in 1981 (De Cock *et al.*, 2011; Sharp and Hahn, 2012). The human immunodeficiency (HI) virus was isolated two years later, and confirmed as the cause of AIDS in 1984 (De Cock *et al.*, 2011; Sharp and Hahn, 2012; De Cock *et al.*, 2012). Little did anyone know that this newly described disease, would become one of the most prominent global pandemics in human history (De Cock *et al.*, 2011; Sharp and Hahn, 2012; De Cock *et al.*, 2012). Approximately 15 years after AIDS was first described, ART was introduced, which represented a major milestone in the history of the disease (De Cock *et al.*, 2012). Since its introduction in (1996) ART quickly revolutionised HIV-care (De Cock *et al.*, 2011; Does *et al.*, 2003). Enormous international efforts followed and are still continuing across the globe to treat PLWH (De Cock *et al.*, 2011; Does *et al.*, 2003).

### **1.4.2. Epidemiology of HIV/AIDS**

Between 1990 and 2010, HIV/AIDS, and the subsequent introduction of ART, both had dramatic effects on the mortality rates in SSA and the rest of the world characterised by an initial increase in the mortality rate due to HIV/AIDS, followed by a substantial reduction in HIV/AIDS-related mortality after the successful introduction of ART, and a subsequent increase in the number of PLWH (Lozano *et al.*, 2013; Houle *et al.*, 2014; Kassebaum *et al.*, 2014). More than 35 million people have died as a result of HIV/AIDS since its discovery (Ruelas and Greene, 2013). It is also estimated that around 2012, 34 million people were living with HIV/AIDS (2.7 million newly infected) and 1.8 million of these people died that year (De Cock *et al.*, 2012).

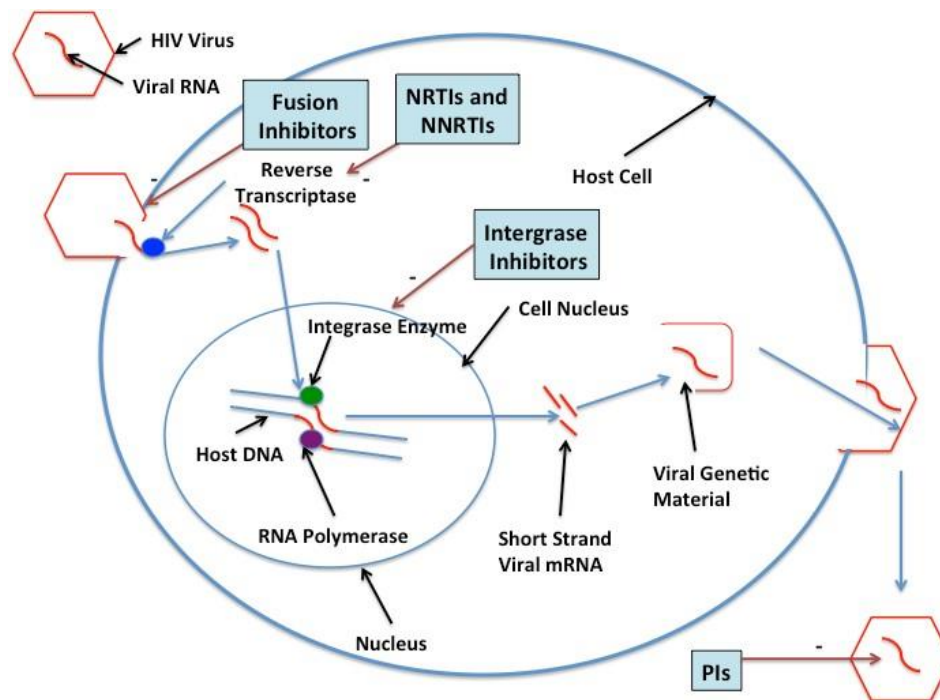
HIV/AIDS remains the leading burden of disease in SSA (22.5 million PLWH in 2009). It is estimated that one in every 20 individuals in SSA are HIV-positive and this represents more than two-thirds of the global HIV/AIDS population (De Cock *et al.*, 2012; Ruelas and Greene, 2013). HIV/AIDS resulted in a 13-year drop in the median life expectancy (49 years for men and 52.5 years for women; 2011) in SA (Levitt *et al.*, 2011; Houle *et al.*,



2014; Dorrington, 2000). Furthermore, SA contributes to approximately 17 % of the global HIV/AIDS population (Houle *et al.*, 2014).

### 1.4.3. The Human Immunodeficiency (HI) Virus (HIV)

HIV is classified as a retrovirus (Ribonucleic acid; RNA) virus that replicates by inserting a deoxyribonucleic acid (DNA) copy of its genome into the host cell (Kirchhoff, 2013; Ruelas and Greene, 2013). After entering the body, the virus starts its lifecycle by binding to a cluster of differentiation-4 (CD4)- receptors and one of two co-receptors that is located on the surface of a CD4<sup>+</sup> T-lymphocyte (**Fig. 1.2**) (Kirchhoff, 2013; Ruelas and Greene, 2013; Does *et al.*, 2003; Stricker, 2003).



**Fig. 1.2.** The Lifecycle of HIV and Role of ART in Blocking Key Steps in the Lifecycle of the HIV. **Abbreviations:** HIV: Human Immunodeficiency Virus; RNA: Ribonucleic Acid; mRNA: Messenger RNA; DNA: Deoxyribonucleic Acid; PI: Protease Inhibitor; NRTI: Nucleoside Reverse Transcriptase Inhibitor; NNRTI: Non-Nucleoside/Nucleotide Reverse Transcriptase Inhibitor. **Symbols:** - = Inhibit (Kirchhoff, 2013; Ruelas and Greene, 2013; Munir *et al.*, 2013; Arts and Hazuda, 2012; Palmisano and Vella, 2011; Sierra and Walter, 2012).

After fusing with the host cell, the virus releases its genetic material into the host cell's cytoplasm (Kirchhoff, 2013; Munir *et al.*, 2013; Does *et al.*, 2003; Stricker, 2003). Subsequently, the HIV reverse transcriptase enzyme converts the single-strand virus RNA into double-strand linear HIV DNA (Kirchhoff, 2013; Munir *et al.*, 2013; Does *et al.*, 2003; Stricker, 2003), which enters the host cell's nucleus and integrates with the host's DNA via integrase enzyme actions (Kirchhoff, 2013; Does *et al.*, 2003; Stricker, 2003). The host cell's RNA-polymerase causes replication of the HIV genomic material, including shorter strands messenger RNA (mRNA) (Kirchhoff, 2013; Stricker, 2003). The mRNA is utilised as a blueprint to generate long-strand HIV proteins (Kirchhoff, 2013; Stricker, 2003). HIV proteases cut the long-strand HIV proteins into smaller HIV proteins (Kirchhoff, 2013; Arts and Hazuda, 2012), which assemble with HIV RNA genetic material leading to the formation of a new HIV-particle (Kirchhoff, 2013).

Finally, the newly formed HIV particle buds off from the host cell taking with it part of the host cell's outer envelope (Kirchhoff, 2013; Arhel, 2010; Stricker, 2003). The surface of the newly formed HIV particle becomes studded with HIV glycoproteins (protein/sugar combinations) that are used to bind to CD4 cell co-receptors to infect other cells (Kirchhoff, 2013).

#### 1.4.4. Anti-Retroviral Therapy (ART)

More than thirty anti-HIV drugs are currently approved globally and in use to fight HIV/AIDS by altering or inhibiting the HI virus' life cycle (Ruelas and Greene, 2013; Arts and Hazuda, 2012; Palmisano and Vella, 2011). ART drugs are designed to block key steps in viral replication and are classified according to their target viral enzymes (**Fig. 1.2**) (Arts and Hazuda, 2012; Palmisano and Vella, 2011; Sierra and Walter, 2012).

Nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) inhibit the key retroviral enzyme called reverse transcriptase and only act once the host cell is infected (Arts and Hazuda, 2012; Palmisano and Vella, 2011; Sierra and Walter, 2012). This group of drugs also includes non-NRTIs (NNRTIs) that block viral DNA-synthesis (Does *et al.*, 2003; Arts and Hazuda, 2012; Sierra and Walter, 2012).

On the other hand, protease inhibitors (PIs) act later in the life cycle of HI virus, by blocking the protease enzyme (involved in the cleavage of the viral polypeptide to produce

functional viral enzymes) (Does *et al.*, 2003; Palmisano and Vella, 2011; Sierra and Walter, 2012). Other ART drug classes furthermore include chemokine receptor antagonists, fusion inhibitors that act on proteins involved in the viral uptake, and integrase-inhibitors that block the enzyme activity of enzymes involved in the integration of viral-DNA into the host cell DNA (Arts and Hazuda, 2012; Palmisano and Vella, 2011; Sierra and Walter, 2012).

Highly active ART (HAART) is the term used to describe a regimen of 3 or more ART-drugs taken simultaneously (Does *et al.*, 2003; Arts and Hazuda, 2012; Palmisano and Vella, 2011). This helps to prevent the virus (highly mutational) from becoming drug resistant (Does *et al.*, 2003; Arts and Hazuda, 2012; Palmisano and Vella, 2011).

## **1.5. HIV/AIDS, ART, Obesity and CVD**

### **1.5.1. Introduction**

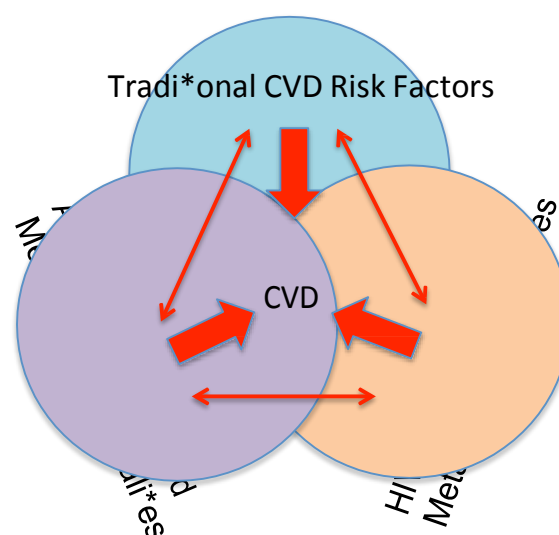
The HI virus *per se*, ART drug class, and duration of ART have all been associated with increased cardiovascular risk (Islam *et al.*, 2012). HIV, ART, and their associated metabolic abnormalities such as dyslipidaemia, lipodystrophy and insulin resistance are also directly and/or indirectly implicated in CVD pathogenesis (Mayosi *et al.*, 2009; Barbaro *et al.*, 2003). HIV- and ART-associated comorbidities are becoming a concern as the duration of exposure to ART and the HI virus parallels the increased exposure to traditional cardiovascular risk factors due to improved longevity seen in HIV-infected populations on ART (Islam *et al.*, 2012; Fedele *et al.*, 2011). HIV and ART seem to increase cardiovascular risk and risk to experience an adverse cardiovascular event (Islam *et al.*, 2012).

Many studies, most notably the *Data collection on Adverse events of Anti-HIV Drugs* (D:A:D) study, found an increased incidence of myocardial infarction (MI) in the HIV-infected study population compared the HIV-negative control group; furthermore, the study population had higher serum levels of cholesterol and TGs compared the HIV-negative control group (Lo and Plutzky, 2012). Furthermore, a recent study found an increased prevalence of subclinical coronary artery disease in young HIV-infected patients (6.5 % had severe obstructive coronary artery disease) with no prior history of CVD compared to

HIV-negative controls who were of similar age, sex, family history, BMI, smoking status, TC and LDL-cholesterol (Lo *et al.*, 2010). In hospital-based studies from SSA, increased incidence of macrovascular arteritis, pulmonary hypertension, cardiomyopathy and tuberculosis-pericarditis were found in HIV/AIDS populations compared to the general population (Mayosi *et al.*, 2009). Carotid intima thickness was found to be higher in HIV patients compared to age-matched HIV-negative controls and was positively associated with HIV-infection and its treatment (Lo *et al.*, 2010; Eira *et al.*, 2012). This evidence of increased cardiovascular risk in PLWH is further underlined by studies that found a higher prevalence of MI in HIV-infected patients compared to non-infected patients (Lo *et al.*, 2010; D'Ascenzo *et al.*, 2012).

### 1.5.2. Factors Associated with Cardiovascular Disease (CVD) in HIV/AIDS

CVD in HIV/AIDS seems to be multifactorial in nature and includes increased traditional cardiovascular risk factors, overlap of risk factors, and the interplay of risk factors such as the virus, HAART, and traditional cardiovascular risk factors (**Fig. 1.3**) (Fedele *et al.*, 2011; Berrueta *et al.*, 2010; Cioe *et al.*, 2014).



**Fig. 1.3.** Cardiovascular Risk Factors in PLWH. **Abbreviations:** CVD: Cardiovascular Disease; HIV: Human Immunodeficiency Virus; ART: Antiretroviral Therapy (Fedele *et al.*, 2011).

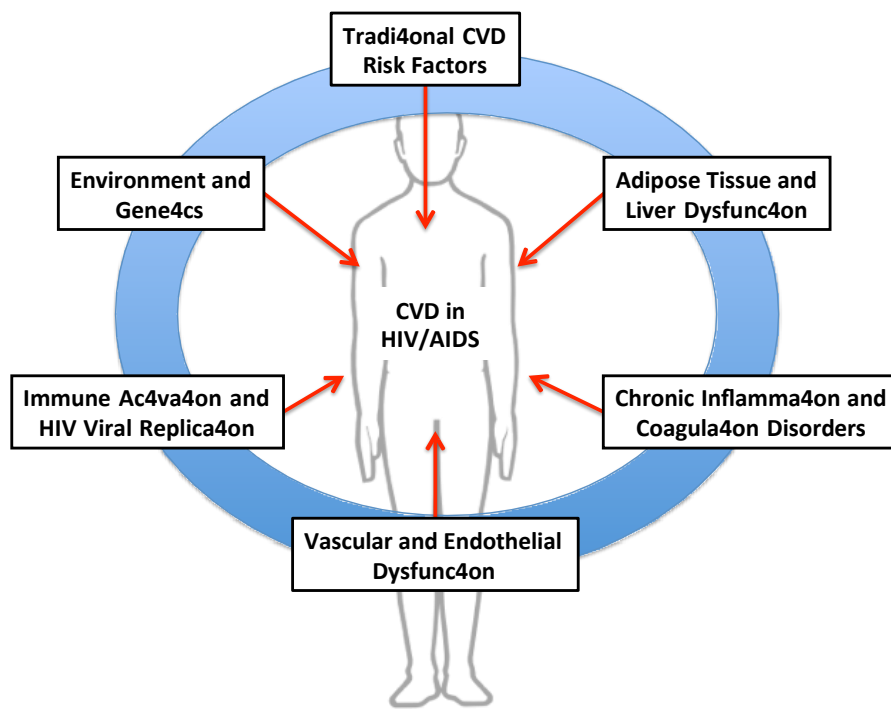
Both HIV-infection and many ART drugs have been associated with detrimental cardiovascular effects (Dubé *et al.*, 2015) and increased cardiovascular risk is in part due to their effects on the body's metabolism, and in part due to direct vascular effects (Torriani *et al.*, 2008; Franciscia *et al.*, 2009; Ismail *et al.*, 2009; Ross *et al.*, 2009) (**Table 1.2**).

**Table 1.2.** *Effects of HIV-Infection and ART on the Vasculature.* **Abbreviations:** EC: Endothelial Cell; ED: Endothelial Dysfunction; HIV: Human Immunodeficiency Virus; ART: Antiretroviral Therapy (Dubé *et al.*, 2015; Chaves *et al.*, 2003; Gil *et al.*, 2003; Lagathu *et al.*, 2014; Obel *et al.*, 2007).

HIV	ART
<ul style="list-style-type: none"> <li>• ED</li> <li>• Lipid Disorders</li> <li>• Viral Protein EC Activation</li> <li>• Systemic inflammation</li> <li>• Direct HIV-infection of the Endothelium</li> <li>• Enhanced Atheroma Formation</li> <li>• Pro-thrombotic State</li> </ul>	<ul style="list-style-type: none"> <li>• ED</li> <li>• Increased EC Permeability</li> <li>• Increased Oxidative Stress</li> <li>• Increased Mononuclear Cell Adhesion</li> <li>• Insulin Resistance</li> <li>• Accelerated Lipid Accumulation in Vessel Wall</li> <li>• Impaired Response to Vascular Injury</li> <li>• Lipodystrophy</li> <li>• Inflammation and Immune Activation</li> </ul>

### 1.5.3. The HI Virus and CVD

An increased incidence of adverse cardiovascular events has been noted in treatment-naïve PLWH when compared to HIV-patients treated with ART, which points to a role for the HI virus in the pathogenesis of CVD (Fedele *et al.*, 2011; Lo *et al.*, 2010; Jiang *et al.*, 2006). Numerous factors associated with the HI virus have been implicated (**Fig. 1.4**).



**Fig. 1.4.** Factors Involved in the Pathogenesis of CVD in PLWH. HIV: Human Immunodeficiency Virus; AIDS: Acquired Immunodeficiency Syndrome; CVD: Cardiovascular Disease (Malaza *et al.*, 2012; Fedele *et al.*, 2011; Lo and Plutzky, 2012; Lo *et al.*, 2010).

It appears that the HI virus can contribute to the pathogenesis of CVD via chronic immune activation, inflammation, and direct viral actions that lead to endothelial dysfunction (ED) and ultimately other CVDs (Malaza *et al.*, 2012; El Assar *et al.*, 2013; Lo *et al.*, 2010). Chronic inflammation is known as a cardiovascular risk factor and has been reported in HIV-infected patients due to the HI virus itself as the HI virus protein, Tat, is known to be involved in the production of ROS and increased apoptosis (Masiá *et al.*, 2007; Wu *et al.*, 2007; Vilhardt *et al.*, 2013).

#### 1.5.4. Antiretroviral Therapy (ART) and CVD

Although HAART considerably improved the survival rate of PLWH, the use of ART has on the other hand also been associated with an increased incidence of cardiovascular risk factors such as insulin resistance, hypertension, dyslipidaemia, lipodystrophy, and diabetes mellitus (Signorini *et al.*, 2012; Islam *et al.*, 2012; Fedele *et al.*, 2011). A study in

2011 found that 26.2 % of HIV patients presented with at least one HAART-associated comorbidity, such as hypertension (16.7 %), vascular diseases (5.6 %), and diabetes mellitus (3.9 %) (Signorini *et al.*, 2012). A systematic review and meta-analysis furthermore found that the relative risk of developing CVD was 1.61-fold higher in HIV-infected without ART patients (HIV-ART) compared to HIV-infected patients on ART-treatment (HIV+ART), and 2-fold higher in HIV-infected patients receiving no ART-treatment (HIV-ART) compared to HIV- and ART-naïve controls (-HIV-ART) (Islam *et al.*, 2012).

Specific ART drug classes have been shown to increase cardiovascular risk per year of exposure (1.05 for PIs, 1.11 for NRTIs and 1.04 for NNRTIs) (Islam *et al.*, 2012). PIs have been linked to the pathogenesis of ED, whereas NRTIs and NNRTIs seem to be less detrimental; however, the specific mechanisms underlying their contribution to CVD remains to be fully elucidated. (Torriani *et al.*, 2008; Dubé *et al.*, 2015; Francisci *et al.*, 2009). Furthermore, findings in studies related to ART and endothelial function often generate contradictory findings (Dubé *et al.*, 2015; Francisci *et al.*, 2009; Kristoffersen *et al.*, 2009).

Nonetheless, ED seems to be the link between ART and CVD (Dubé *et al.*, 2015; Francisci *et al.*, 2009). The pathogenesis of ART-induced ED appears to be related to decreased nitric oxide (NO) production through reduction in nitric oxide synthase (NOS) expression and the increased production of ROS that reduces bioavailability of antioxidants (Dubé *et al.*, 2015; Masiá *et al.*, 2007; Mondal *et al.*, 2004). Increased hydrogen peroxide levels have been positively correlated to increased C-reactive protein and LDL cholesterol levels (Masiá *et al.*, 2007). Also, hydrogen peroxide levels were found to be significantly lower in patients receiving NNRTIs compared to PIs (Masiá *et al.*, 2007).

Although data from developed countries shows an association between ART and increased risk of developing premature CVD and experiencing cardiovascular events, population-based studies on the effects and outcomes of ART in populations from SSA are scarce (Malaza *et al.*, 2012; Islam *et al.*, 2012).



### 1.5.5. Obesity in HIV/AIDS and CVD

Improved life expectancy and the subsequent dramatic increase in the number of PLWH (more than 33 million globally), contribute to the emergence of NCDs such as CVD in HIV populations (Islam *et al.*, 2012; Fedele *et al.*, 2011). Furthermore, increased exposure to general NCD and cardiovascular risk factors (also experienced by the general population) in PLWH (due to improved longevity) is an emerging challenge (Islam *et al.*, 2012; Fedele *et al.*, 2011). Many studies found that the prevalence of traditional cardiovascular risk factors in PLWH is high and in some cases even higher than the general population (Fedele *et al.*, 2011). However, the relative contribution of each cardiovascular risk factor to the pathogenesis of CVD in PLWH seems to differ from the general population and suggest a more complex interplay between the disease, treatment, and traditional cardiovascular risk factors (Fedele *et al.*, 2011).

HIV/AIDS and obesity are both major public health concerns (Keithley *et al.*, 2009). Although ART can reverse HIV-associated weight loss, the emergence of obesity in PLWH is becoming of greater concern (Amarosa *et al.*, 2005; Lakey *et al.*, 2013; Taylor *et al.*, 2014).

Studies have shown that the prevalence of obesity in HIV populations is either approaching or mimicking that of the general population (Amarosa *et al.*, 2005; Wanke *et al.*, 2000). A study in the USA found that 63 % of HIV-infected participants was overweight/obese (Crum-Cianflone *et al.*, 2008). Another study showed that 49 % of the HIV-infected study population was overweight/obese while other estimates indicate 30 % to 40 % of the HIV-infected population in the USA to be obese (Leite and de Mattos Marinho Sampaio, 2008).

It was furthermore found that HIV/AIDS and ART influences adipose tissue biology and distribution (Giralt *et al.*, 2011; Grima *et al.*, 2010). Lipodystrophy that includes peripheral adipose tissue atrophy and/or visceral fat hypertrophy has been reported and is estimated to affect about half of HIV/AIDS patients on treatment (including children) (Giralt *et al.*, 2011; Arpadi *et al.*, 2009). One study found that 45.7 % of its study population presented with central obesity (Jaime *et al.*, 2006). NRTIs, NNRTIs, and PIs have all been implicated in lipodystrophy (Giralt *et al.*, 2011; Mallon *et al.*, 2003; Anuurad *et al.*, 2010). These complications are becoming increasingly relevant as HIV populations on ART are aging and becoming treatment experienced (Lo and Plutzky, 2012; Gupta *et al.*, 2012; Bertisch *et al.*, 2011).



## **1.6. HIV/AIDS: The South African (SA) Context**

### **1.6.1. Introduction**

Soon after the discovery of HIV, two types of HIV were identified (De Cock *et al.*, 2011; De Cock *et al.*, 2012). HIV type-1 (HIV-1; a virus that is thought to have crossed the animal (chimpanzees) to human barrier in Western Africa) was identified as the predominant cause of the HIV/AIDS epidemic the world and also in SA (De Cock *et al.*, 2011; Sharp and Jaffe, 2012; De Cock *et al.*, 2012). HIV type-2 (HIV-2) was identified soon after HIV-1 (also thought to have originated in Western Africa), but is rarely found outside central Africa (De Cock *et al.*, 2011; Sharp and Jaffe, 2012; De Cock *et al.*, 2012).

SA has the largest HIV/AIDS population and Government-sponsored ART roll-out programme in the world (Vermund, 2013; Houle *et al.*, 2014; Hontelez *et al.*, 2013). SA is furthermore in the midst of epidemiological transition that involves a shift in pattern of disease from more prevalent pre-transitional infectious diseases to more affluent NCDs such as CVD, due to factors such as economic growth, urbanisation, and westernization (Mayosi *et al.*, 2009; Fedele *et al.*, 2011; Beleta *et al.*, 2014). Also, more affluent NCD risk factors such as an unhealthy diet that is high in fat and sugar and a sedentary lifestyle are becoming more prevalent nationwide (Mayosi *et al.*, 2009; Fedele *et al.*, 2011).

### **1.6.2. South Africa's First-Line ART Fixed Drug Combination (FDC)**

The SA Government's new, once daily, first-line ART fixed drug combination (FDC) was implemented in April 2013 and prescribed to millions of HIV-infected patients (Southern African H. I. V, 2013). It consists of two NRTIs (Emtricitabine (FTC): 200 mg / day; Tenofovir disoproxil fumarate (TDF): 300 mg / day) and a NNRTI (Efavirenz (EFV): 600 mg / day) (Southern African H. I. V, 2013; South African Antiretroviral Guidelines, 2015; Meintjes *et al.*, 2014). A once daily FDC decreases the burden of patients subjected to a multiple tablet regime and improves treatment adherence (Meintjes *et al.*, 2014). HIV-positive patients in SA with a CD4<sup>+</sup> count of < 500 cells /  $\mu$ l are placed on ART (South African Antiretroviral Guidelines, 2015 (available at [www.doh.gov.za](http://www.doh.gov.za))).

### **1.6.2.1. Emtricitabine (FTC)**

FTC (molecular weight: 247.2 g / mol) is a NRTI that is a synthetic nucleoside analogue of cytidine (HIV Druginteractions, 2011). Cellular enzymes phosphorylate FTC to FTC-5'-triphosphate which in turn competes with the natural substrate, deoxycytidine 5'-triphosphate and is subsequently incorporated into the nascent viral DNA and leads to chain termination (Arts and Hazuda, 2012; Meintjes *et al.*, 2014; HIV Druginteractions, 2011). The recommended daily allowance for FTC is 300 mg / day (Meintjes *et al.*, 2014).

### **1.6.2.2. Tenofovir Disoproxil Fumarate (TDF)**

TDF (molecular weight: 287.2 g / mol) is also a NRTI and an acyclic nucleoside phosphonate diester analogue of adenosine monophosphate (AMP) and requires initial diester hydrolysis to convert to TDF before being phosphorylated by cellular enzymes and subsequently converted into TDF diphosphate (Arts and Hazuda, 2012; Palmisano and Vella, 2011; Sierra and Walter, 2012). TDF diphosphate inhibits HIV-1 reverse transcriptase through competition with the natural substance deoxyadenosine 5'-triphosphate and leads to DNA chain termination after incorporation into the viral-DNA (Does *et al.*, 2003; Arts and Hazuda, 2012; Palmisano and Vella, 2011). The recommended daily allowance for TDF is 300 mg / day (Meintjes *et al.*, 2014).

### **1.6.2.3. Efavirenz (EFV)**

EFV (molecular weight: 315.7 g / mol) is a NNRTI that is selective for HIV-1 and diffuses into the host cell and binds next to the active site of the reverse transcriptase enzyme (Palmisano and Vella, 2011; Sierra and Walter, 2012; Meintjes *et al.*, 2014). This binding results in a conformational change in the enzyme that inhibits its function (Does *et al.*, 2003; Arts and Hazuda, 2012; Palmisano and Vella, 2011). It is further a non-competitive inhibitor of HIV-1 reverse transcriptase with respect to template, primer or nucleoside triphosphates (Does *et al.*, 2003; Arts and Hazuda, 2012). The recommended daily allowance for EFV is 300 mg / day administered at night (Meintjes *et al.*, 2014).

## 1.7. Conclusion

The emergence of CVD as a major contributor to mortality and morbidity in PLWH appears to be multifactorial in nature (Hsue *et al.*, 2004; Fedele *et al.*, 2011; Sudano *et al.*, 2006). The HI virus itself contributes to the CVD continuum through its effect on the serum lipid profile and direct infection of vascular ECs (Hsue *et al.*, 2004; Fedele *et al.*, 2011; Sudano *et al.*, 2006). On the other hand, it is suggested that ART can reduce cardiovascular risk in PLWH though suppressing viral replication, but is also associated with cardiovascular risk through its many associated metabolic abnormalities such as insulin resistance, diabetes mellitus, dyslipidemia, and lipodystrophy (Signorini *et al.*, 2012; Fedele *et al.*, 2011; Behrens *et al.*, 2003). Although the mechanism through which ART directly contributes to the pathogenesis of CVD is unknown, an increased incidence for CVD events and disorders has been found to be associated with certain combinations of drugs and drug-classes (Hsue *et al.*, 2004; Sudano *et al.*, 2006; Calza *et al.*, 2008).

Furthermore, improved longevity and quality of life in PLWH, due to the success of HAART, manifests with increased traditional cardiovascular risk factors similar to those seen in the general population (Hsue *et al.*, 2004; Fedele *et al.*, 2011). Obesity in particular is becoming increasingly prominent in HIV-populations, as studies have found rates of overweight and obesity to approach or mimic that of the general population (Amarosa *et al.*, 2005; Crum-Cianflone *et al.*, 2008; Keithley *et al.*, 2009). Obesity is a well-known cardiovascular risk factor and comorbidities such as lipodystrophy, insulin resistance, and dyslipidemia are shared with ART (Keithely *et al.*, 2009; Lavie *et al.*, 2009; Therchof and Després, 2013). However, the mechanistic interplay between factors including obesity, HAART, and their risk factor profiles in PLWH is not well understood (Crum-Cianflone *et al.*, 2008; Keithley *et al.*, 2009; Lakey *et al.*, 2013).

This interplay between risk factors is of great concern in countries such as SA where obesity, HIV/AIDS and ART-use are highly prevalent and on the rise in conjunction with epidemiological transition and the emergence of traditional cardiovascular risk factors (Norman *et al.*, 2006; Levitt *et al.*, 2011; Mayosi *et al.*, 2009). Taking all these factors in consideration, it is of great importance that the cardiovascular effects of SA's first line ART in the context of obesity is investigated as it could potentially affect millions of lives.

## **1.8. Problem Identification and Aims of the Study**

### **1.8.1. Problem Identification**

The SA Government recently introduced a new first line ART FDC (Odimune®) (Southern African H. I. V, 2013; South African Antiretroviral Guidelines, 2015; Meintjes *et al.*, 2014). Although the precise effects and mechanisms of ART on the cardiovascular system are not well understood, many comorbidities are associated with the use of ART and are impacting CVD outcomes (Fedele *et al.*, 2011). Increasing numbers of overweight/obesity have been reported in HIV-infected populations (Amarosa *et al.*, 2005; Wanke *et al.*, 2000; Keithley *et al.*, 2009; Taylor *et al.*, 2014). The complex interplay between HIV, ART, and traditional cardiovascular risk factors such as obesity needs further investigation (Fedele *et al.*, 2011).

Therefore, the overall aim of this study was to investigate the cardiovascular and metabolic effects of the first line FDC used in SA in the context of obesity.

### **1.8.2. Main Aim of the Study**

To investigate the cardiovascular effects of SA's new first line ART FDC in a rat model of obesity: an *in vivo* and *ex vivo* approach.

### **1.8.3. Specific Aims of the Study**

- a. To induce obesity by feeding a high fat/sucrose diet (HFD) to male Wistar rats.
- b. To determine the effects of ART on cardiometabolic parameters in obese/lean age matched male Wistar rats.
- c. To determine the effects of ART in isolated hearts from obese and lean age matched male Wistar rats subjected to ischaemia-reperfusion injury.
- d. To determine the effects of ART on the expression and phosphorylation of important vascular signaling proteins in cardiac tissue from baseline and ischemia-reperfusion hearts.

## Chapter 2 - Materials and Methods

### 2.1. Materials

- *AEC Amersham* (Buckinghamshire, United Kingdom): Enhanced chemiluminescence (ECL) detection reagent, and Horseradish peroxidase-linked anti-rabbit immunoglobulin G (IgG; Secondary antibody).
- *Afrox Ltd.* (Johannesburg, Gauteng, SA): 5 % CO<sub>2</sub> / 95 % O<sub>2</sub> (To gas the perfusate).
- *Bayer (Pty) Ltd.* (Isando, Gauteng, SA): Eutha-naze (Sodium pentobarbitone; C<sub>11</sub>H<sub>17</sub>N<sub>2</sub>NaO<sub>3</sub>)
- *BIO-RAD Inc.* (Hercules, California, USA): Chemidoc<sup>TM</sup> MP Imager System with Image Lab<sup>TM</sup> - 5 software.
- *Cell Signaling Technologies* (Beverly, Massachusetts, USA): Antibodies for the detection of: Total- and phosphorylated eNOS; Total- and phosphorylated PKB/Akt, Total- and phosphorylated AMPK; IκBα, and β-Tubulin.
- *Cipla MedPro (Pty) Ltd.* (Bellville, Western Cape, SA): Odimmune® Tablet (SA's first-line antiretroviral FDC), GlucoPlus<sup>TM</sup> (Conventional glucose meter).
- *Greiner Bio-One Inc.* (Monroe, North Carolina, USA): Cryo tubes.
- *Merck (Pty) Ltd.* (Darmstadt, Germany): Sodium chloride (NaCl), Sodium bicarbonate (NaHCO<sub>3</sub>), Potassium chloride (KCl), Potassium dihydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>), Magnesium sulphate heptahydrate (MgSO<sub>4</sub>·7H<sub>2</sub>O), Sodium Sulfate (NaSO<sub>4</sub>), Calcium chloride dihydrate (CaCl<sub>2</sub>·2H<sub>2</sub>O), D(+)-Glucose anhydrous (C<sub>6</sub>H<sub>12</sub>O<sub>16</sub>), 2,3,5-Triphenyltetrazolium chloride (TTC; C<sub>19</sub>H<sub>15</sub>ClN<sub>4</sub>), Sodium dihydrogen phosphate dehydrate (NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O), Disodium hydrogenorthophosphate (Na<sub>2</sub>HPO<sub>4</sub>), Formaldehyde (CH<sub>2</sub>O), Sodium pyrophosphate (Na<sub>4</sub>O<sub>7</sub>P<sub>2</sub>), Acrylamide (C<sub>3</sub>H<sub>5</sub>NO), Methanol (CH<sub>4</sub>O), Glycine (NH<sub>2</sub>CH<sub>2</sub>COOH), and Tris (hydroxymethyl aminomethane), Hydrochloric acid (HCl), Glycerol (C<sub>3</sub>H<sub>8</sub>O<sub>3</sub>), Polyvinylidene difluoride (PVDF) membrane (Immobilon<sup>TM</sup>-P), Enzyme-linked immunosorbent assay (ELISA) kit for rat/mouse insulin (96 well plate assay; Cat. EZRMI-13K), Ethanol (C<sub>2</sub>H<sub>6</sub>O), phosphoric acid (H<sub>3</sub>PO<sub>4</sub>).
- *Roche Diagnostics (Pty) Ltd.* (Cape Town, Western Cape, SA): Bovine serum albumin (BSA).

- *Santa Cruz Biotechnologies Inc.* (Santa Cruz, California, USA): Antibodies for the detection of: NADPH oxidase sub-unit p22 Phox, and Nitrotyrosine.
- *Sigma* (St. Louis, MO, USA): Evans blue ( $C_{34}H_{24}N_6Na_4O_{14}S_4$ ), Phenylmethylsulfonyl fluoride (PMSF;  $C_7H_7FO_2S$ ), Ethylenediaminetetraacetic acid (EDTA;  $C_{10}H_{16}N_2O_8$ ),  $\beta$ -glycerophosphate ( $C_3H_7Na_2O_6P$ ), Sodium orthovanadate ( $Na_3VO_4$ ), Sodium dodecyl sulfate (SDS;  $C_{12}H_{25}NaO_4S$ ), Triton<sup>®</sup> X-100 ( $C_{18}H_{28}O_5$ ), Mercaptoethanol ( $C_2H_6OS$ ), Ponceau red ( $C_{18}H_{14}N_2Na_2O_7S_2$ ), Ammonium persulfate (APS;  $H_8N_2O_8S_2$ ), Commassie brilliant blue ( $C_{37}H_{34}N_2Na_2O_9S_3$ ), Aprotinin ( $C_{284}H_{432}N_{84}O_{79}S_7$ ), Leupeptin ( $C_{20}H_{38}N_6O_4$ ), Polyethylene glycol sorbitan monolaurate (Tween<sup>®</sup> 20;  $C_{58}H_{114}O_{26}$ ), and 1,2-Bis(dimethylamino)ethane (TEMED), Bromophenol blue ( $C_{19}H_{10}Br_4O_5S$ ).
- *Thermo Scientific* (Lithuania, European Union): PageRuler Prestained Protein Ladder (Molecular marker for Western blot).
- *The Scientific Group (Pty) Ltd.* (Milnerton, Western Cape, RSA): SGVac tubes (Blood collection tubes for serum, containing clot activator and serum separating gel).

## **2.2. Methods**

### **2.2.1. Ethics Clearance**

The study was approved by The Research Ethics Committee: Animal Care and Use of the University of Stellenbosch (US; Faculty of Medicine and Health Sciences; Protocol Number: SU-ACUM13-00025). The animal handling and care throughout the study complied with the accepted standards for the use of animals in research and teaching as reflected in the South African National Standards document (SANS 10386: 2008, available at [www.sun.ac.za/research](http://www.sun.ac.za/research)) of the South African Bureau of Standards (SABS).

### **2.2.2. Infrastructure and Expertise**

The research study was performed at the Division of Medical Physiology, US. All the required materials, techniques, facilities, and expertise were established and available for the successful completion of this study. There was access to an animal care facility (Accredited by the Association for Assessment and Accreditation of Laboratory Animal Care), a heart perfusion laboratory, a Western blot laboratory, and appropriate storage facilities for all samples. For specialized biochemical analysis, samples were analyzed by Dr Blackhurst of the Division of Chemical Pathology at University of Cape Town (UCT).

### **2.2.3. Animal Selection and Care**

Age matched male Wistar rats (Initial weight range: 151 g – 272 g) were used for all experimental purposes. The US (Faculty of Medicine and Health Sciences) animal care facility on campus provided, housed, and cared for all the animals during the whole study. All the animals were housed (Maximum 4 animals / cage) in a properly controlled environment (22 °C; 40 % humidity; 12 hour artificial day / night cycle) during the course of this study.

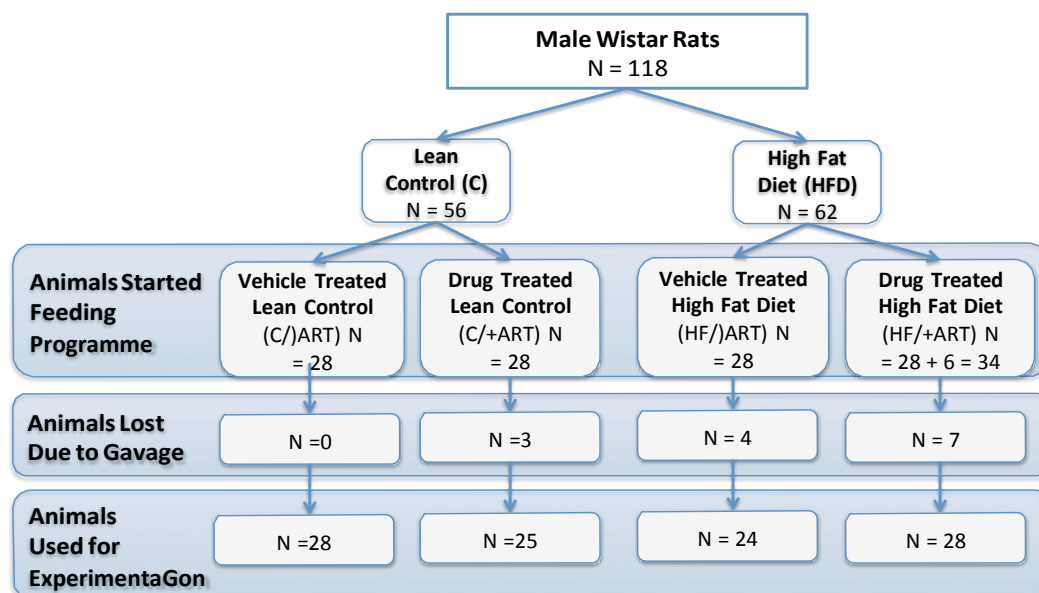
The drug suspension and vehicle (water) were administered to the rats via oral gavage once daily during the 6-week ART-treatment programme by a qualified staff member (Mr. Noël Markgraaff; Manager: Care and Use of Laboratory Animals, SU). The procedure of

gavaging involved the insertion of a feeding tube along the roof of the animal's oral cavity towards the animal's left side and down the animal's esophagus and into the stomach of the animal. The ART-suspension (ART-drug suspended in water) or water (for vehicle treated groups) was therefore injected directly in the stomach of the animal.

#### 2.2.4. Experimental Design, Groups and Sample Sizes (N)

Animals were randomly divided into lean control and HFD groups. The lean- and HFD groups were further randomly subdivided into antiretroviral treatment (+ART) and vehicle treated (-ART) groups. Therefore, the total number of study groups was as follows (**Fig. 2.1**):

- A vehicle treated lean control group (C/-ART),
- A drug treated lean control group (C/+ART),
- A vehicle treated HFD group (HF/-ART),
- A drug treated HFD group (HF/+ART).



**Fig. 2.1.** Experimental Study Design and Groups. **Abbreviations:** N: Sample Size; C: Lean Control; HFD: High Fat/Sucrose Diet; +ART: ART Treated; -ART: Vehicle Treated.

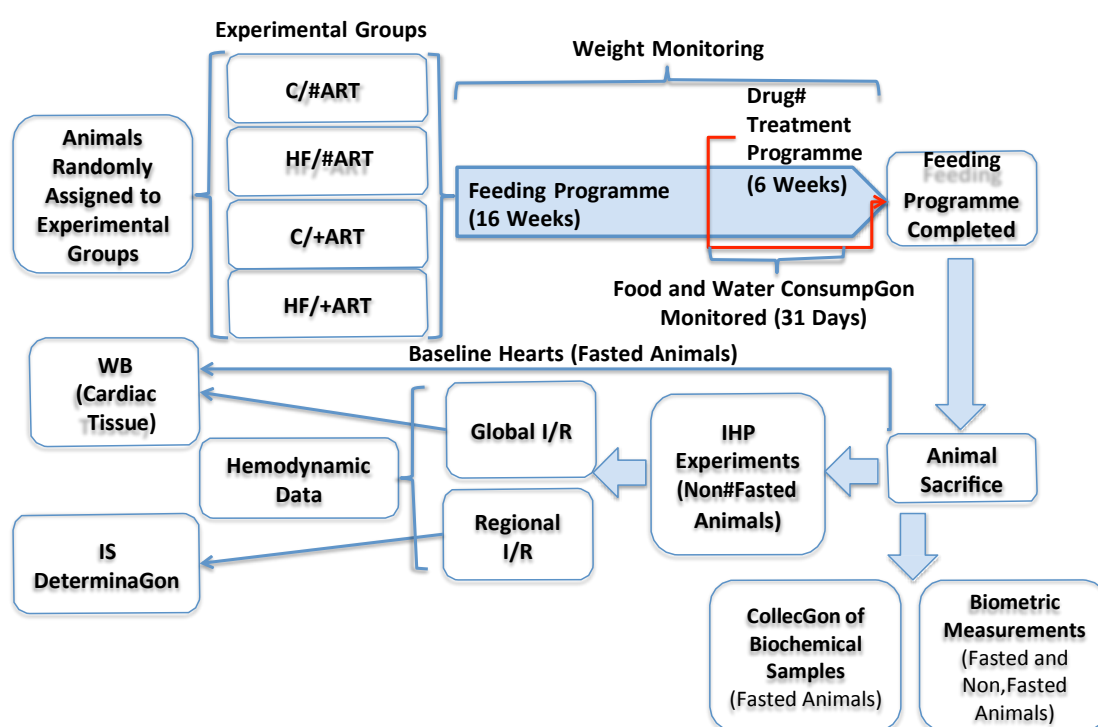
A total number of 118 animals (N = 56 Lean control animals + N = 62 HFD animals) started the feeding programme. A total number of 28 animals were randomly assigned to



each experimental group. An additional HF/+ART group of 6 animals was added to compensate for animal-loss due to gavage (**Fig. 2.1**).

## 2.2.5. General Overview of the Study and Methods Used

At the start of this randomised control study, animals were randomly assigned to 4 experimental groups (**Section 2.2.4**) (**Fig. 2.2**). The experimental groups were placed on a 16-week feeding programme (**Section 2.2.6**) where all the animals followed an *ad libitum* diet (Free access to normal rat chow and water).



**Fig. 2.2.** General Overview of the Investigations. **Abbreviations:** IHP: Isolated Heart Perfusion; I/R: Ischaemia Reperfusion; WB: Western Blot; IS: Infarct Size; C: Lean Control; HFD: High Fat/Sucrose Diet; +ART: Antiretroviral Treated; -ART: Vehicle Treated.

Additionally to the normal rat chow and water, the HFD groups (HF/-ART and HF/+ART) received free access to high-calorie food (**Section 2.2.7**), which aimed to induce obesity and insulin resistance. This HFD-induced model of obesity in rats has previously been established and characterised in our laboratory (Salie *et al.*, 2014). During the feeding programme, the general condition of the animals was monitored daily and TBM recorded weekly.

Animals were placed on a feeding programme at the age of five to eight weeks (mean initial TBM:  $199.2 \pm 8.18$  g), which lasted a total of 16 weeks. The drug-treatment regime (**Section 2.2.8**) commenced at the onset of week 11 of the feeding programme and continued for the remaining 6 weeks of the feeding programme. The drug dosage for each specific group was calculated (**Section 2.2.9**) according to the average TBM recorded weekly and administered (suspended in water) by means of oral gavage (**Section 2.2.8**). Vehicle-treated groups (C/-ART and HF/-ART) received only water in the same volume as drug-treated groups (C/+ART and HF/+ART), also by means of gavage. During the first 31 days of the drug-treatment programme, the food- and water consumption of all the experimental groups were monitored daily (**Section 2.2.8**).

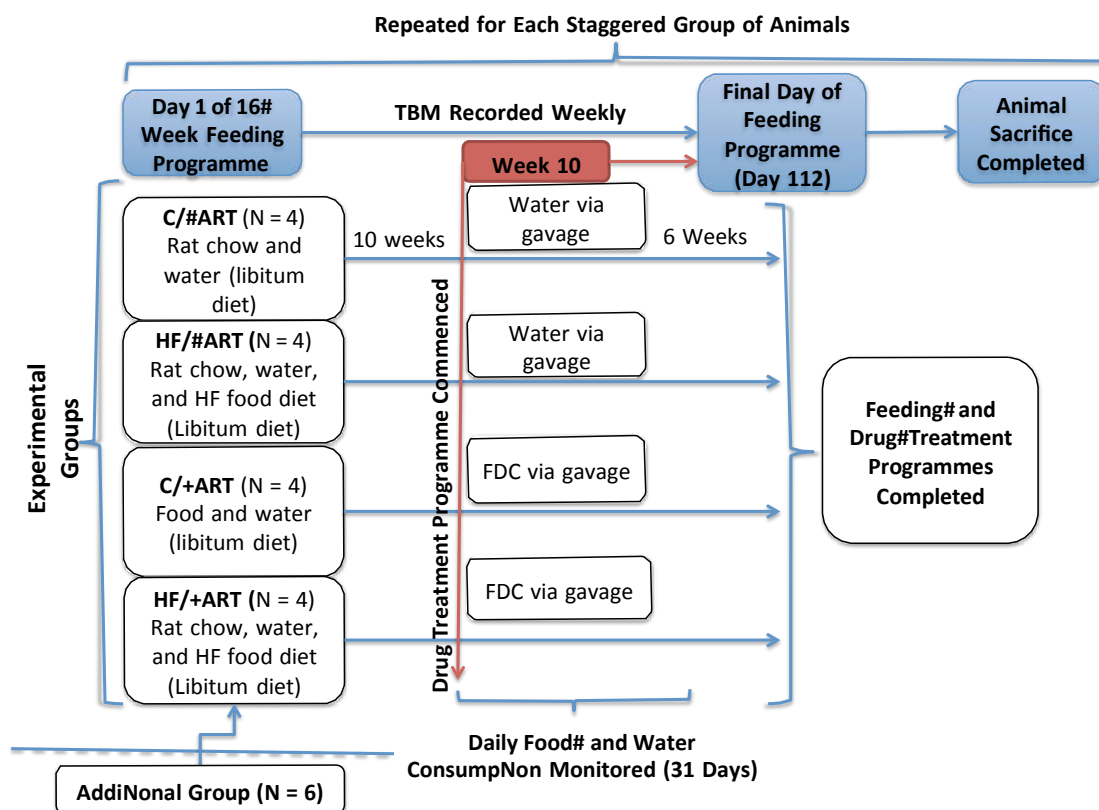
At the end of the feeding programme, animals were randomly sacrificed by means of euthanasia (**Section 2.2.11**) and biometric measurements (all animals) (**Sections 2.2.12 & 2.2.13**) and biochemical samples (fasted animals) (**Section 2.2.14**) were taken. Glucose (directly after anaesthesia) and insulin levels (from stored serum at a later stage) of fasted animals were also measured and recorded. Hearts were excised (**Section 2.2.12**) and either snap-frozen and stored in liquid nitrogen for subsequent Western blot analyses (**Section 2.2.16**) or used for isolated heart perfusion experiments (**Section 2.2.15**). Isolated heart perfusion protocols included global ischaemia for functional analysis or regional ischaemia for infarct size determination (**Section 2.2.15.4**). Hearts were also snap-frozen after the global isolated heart perfusion protocol for later Western blot analysis (**Section 2.2.16**).

## 2.2.6. Feeding Programme

At the end of the study, seven staggered groups completed the feeding & drug treatment programme (**Fig.e 2.3**). An additional 8<sup>th</sup> staggered group consisting of only HFD, drug-treated, animals (HF/+ART; N = 6) was added during the course of study.

The randomly assigned animals started the 16-week feeding- and drug treatment programme in staggered groups consisting of 16 animals (N = 4 animals / experimental group). The staggered groups were separated by at least 2-week intervals to allow sufficient time to complete the necessary laboratory procedures that followed the animal

sacrifice (biometric measurements, serum collection for biochemical analyses, glucose level determinations, and isolated heart perfusion experiments).



**Fig. 2.3.** *Feeding- and Drug-Treatment Programmes.* **Abbreviations:** C: Lean Control; HF: High Fat Diet; +ART: Antiretroviral Treated; -ART: Vehicle Treated; TBM: Total Body Mass; FDC: Fixed Drug Combination; N: Sample Size.

As mentioned previously, all the animals were individually weighed and monitored for any observable changes in their health during the feeding- and drug treatment programme. The initial total body mass of each animal was recoded on the day the feeding programme commenced (Day 1) and continued to be measured weekly (7-day cycles) until the final day of the feeding- and drug treatment programme (Day 112). A final total body mass measurement was recorded on the day the animal was sacrificed.

### 2.2.7. Diet and HFD Food Preparation

The animals followed an *ad libitum* diet throughout the feeding programme (112 days), up until the day of sacrifice (< 2 weeks following the 16-week feeding programme). The diet consisted of standard rat chow and tap water for all the experimental groups.

The HFD experimental groups (HF/-ART and HF/+ART) had additionally free access to a special mixture of HFD food that was prepared and placed separately in each relevant cage, and replaced daily.

The HFD was prepared with Holsum™ butter, condensed milk and normal rat chow and was high in fat, carbohydrate, and sucrose content compared to normal rat chow (**Table 2.1**) (See **Appendix A** For the method of HFD food preparation and nutritional values of Holsum™ butter and condensed milk).

**Table 2.1.** Composition of the Normal Rat Chow and HFD (Salie *et al.*, 2014).

	Lean Diet (Standard Rat Chow)	High Fat Diet
<b>Total Fat (g / 100 g)</b>	4.8	11.5
<b>Saturated Fat (g / 100 g)</b>	0.9	7.6
<b>Cholesterol (mg / 100 g)</b>	2.2	13
<b>Protein (%)</b>	17.1	8.3
<b>Carbohydrate (%)</b>	34.6	42
<b>Sucrose (%)</b>	5.3	20.4

### 2.2.8. Drug Treatment Programme

At the beginning of Week 11 of the 16-week feeding programme, drug administration was initiated and continued for the remaining 6 weeks alongside the feeding programme (**Fig. 2.3**). The drug-treated animals received Odimmune®, which is a commercially available fixed-dose combination ART drug. Odimmune® was obtained on a prescription from a pharmacy and contained the active ingredients EFV, FTC, and TDF.

The dosage of Odimmune® was determined specifically for each treated group of animals (HF/+ART and C/+ART) and prepared weekly by calculations based on the weekly average TBM (mg / kg) of animals in each respective group (**Section 2.2.9**). Staff at the animal care facility administered the correct drug dosage as a water suspension daily by means of oral gavage (see **Fig. 2.3**).

#### **2.2.9. Drug Preparation and Dosage Calculations**

Odimmune® was purchased as tablets containing the human recommended daily dose of active ingredients (600 mg EFV, 200 mg FTC, and 300 mg TDF) for an average person (70 kg). Tablets were grounded to a fine powder, weighed, and the proper mass of powder for each respective drug-treated group calculated weekly and weighed according to the average total body mass of the group. The appropriate dosage (dose / cage) was adjusted weekly according to the respective change in average total body mass of each drug-treated group (See **Appendix B** for Method of Drug Dose Calculation).

#### **2.2.10. Animal Relocation for Laboratory Procedures**

After completing the feeding- and drug-treatment programmes, the animals in each staggered group were weighed and transferred to the animal care facility of the Division of Medical Physiology as needed for experiments and housed temporarily until the day of sacrifice (about 2 animals / experimental group = 8 animals / week). This had to be done in view of logistical reasons. Care was taken to keep this interim relocation period as short as possible (< 1 weeks). During the interim period, the HFD feeding program continued in order to avoid metabolic changes (due to a change in diet) until the day of sacrifice.

#### **2.2.11. Euthanasia**

The animals were anesthetized (IP injection of 160 mg / kg sodium pentobarbitone in the lower right abdomen). Deep anesthesia was confirmed by the disappearance of the pedal pain withdrawal reflex and absence of eye reflexes. Euthanasia was achieved by means

of a clamshell thoracotomy and eventually exsanguination of the heart. After euthanasia, biometric measurements were performed (TBM, heart-, liver-, and IP fat mass).

#### **2.2.12. Biometric Measurements**

To determine the heart mass, the heart of each animal was quickly excised through a clamshell thoracotomy. After the heart was excised, excess tissue was removed from the heart and rapidly weighed in a weighing boat on an electronic scale. Finally, the liver and all the IP fat (from the abdominal cavity) of each animal were also excised and weighed in a weighing boat on an electronic scale.

#### **2.2.13. Glucose Level Determinations**

The blood glucose levels of fasted- and non-fasted anesthetized animals were determined from blood obtained from a prick of the tail's dorsal vein with a sharp needle, using a commercially purchased glucometer (GlucoPlus™). This was performed while the animal was still under deep anesthesia.

#### **2.2.14. Serum Collection and Analysis**

One or two animals from each experimental group were randomly fasted overnight (16- to 24 hours) and serum obtained from blood collected from the thoracic cavity after the heart was excised (**Section 2.2.12**). Serum samples were stored at -80 °C for subsequent analyses of TC-, TG-, HDL cholesterol, conjugated diene (CD)- and Thiobarbuturic Acid Reactive Substance (TBARS) levels by Dr Blackhurst of the Division of Chemical Pathology at UCT. Insulin levels were determined by means of a commercially available ELISA assay kit for rat/mouse insulin levels, purchased from Merck (see **Appendix C** for the principal of the ELISA Assay).

## 2.2.15. Isolated Heart Perfusions

### 2.2.15.1. Pre-Ischaemic Stabilisation on the Isolated Heart Perfusion System

After the heart was excised it was immediately placed in ice-cold Krebs-Henseleit buffer to arrest and decrease cardiac metabolism until it was mounted onto the perfusion system. An *ex vivo* isolated heart perfusion system with circulating, pre-heated (36.8 °C) Krebs-Henseleit buffer (perfusate) that was continuously gassed with 5 % CO<sub>2</sub> and 95 % O<sub>2</sub> through a sintered glass oxygenator (to keep the pH at 7.4) was used for all ischaemia-reperfusion experiments (Bell *et al.*, 2011).

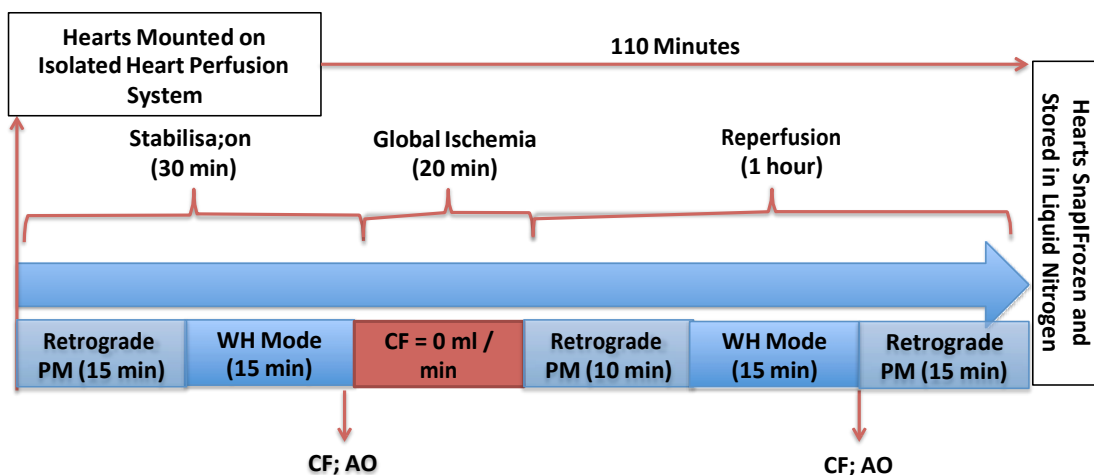
All the hearts were normothermically perfused at 36.8 °C with a preload of 15 cm H<sub>2</sub>O hydrostatic pressure and a left ventricle ejection afterload of 100 cm H<sub>2</sub>O hydrostatic pressure.

Hearts were mounted on the isolated heart perfusion system. After aortic cannulation, each heart was securely tied and retrogradely perfused in Langendorff mode (non-recirculating manner; opposite to normal physiological flow) for the first 15 minutes of the 30-minutes stabilisation period in order to allow atrial perfusion. During this 15-minute period of stabilisation, the left atrium was cannulated and a temperature probe (Thermistor) was inserted into the left coronary sinus through a small incision in the right ventricular outflow tract of the pulmonary artery. This was done in order to monitor, control, and keep the myocardial temperature constant at 36.8 °C throughout the whole experimental protocol. Following the initial 15 minutes of stabilisation, the perfusion system's mode was switched to working-heart mode for a further 15 minutes of stabilisation. After the full stabilisation period of 30 minutes, each heart was subjected to either global- or regional ischaemia-reperfusion protocols (**Sections 2.2.15.2 and 2.2.15.3**).

### 2.2.15.2. Global Ischaemia-Reperfusion

After the period of stabilization (30 minutes), the heart was subjected to global ischaemic injury (Zero-flow: Coronary flow (CF) rate = 0 ml / min) for 20 minutes (**Fig. 2.4**) by the cessation of perfusate (Krebs-Henseleit buffer) supply through both the aortic- and the

ventricular cannulas and kept at a constant temperature of 36.8 °C. The 20-minute period of ischaemia was selected after considering the time it takes for ischaemia to have effect on all proteins of interest and as suggested in literature (Bell *et al.*, 2011).



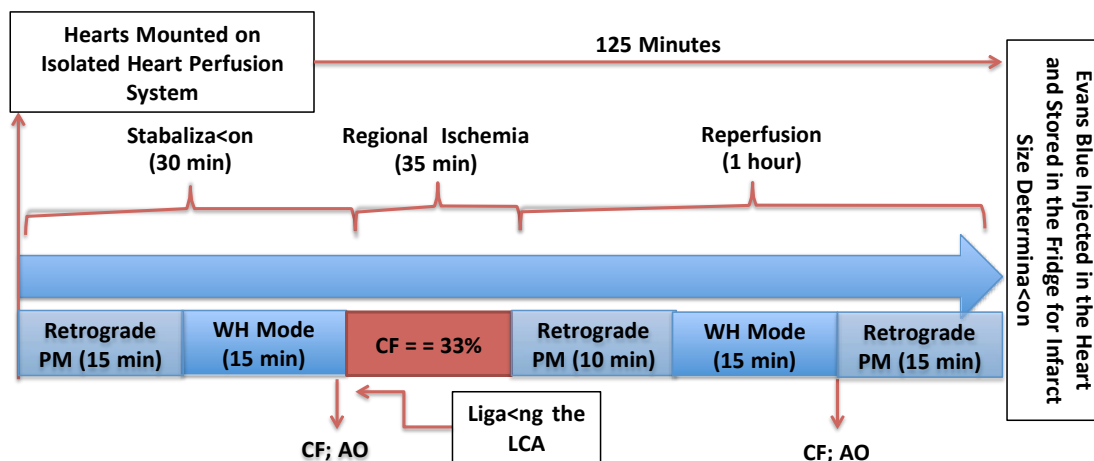
**Fig. 2.4.** Protocol for Global Ischaemia-Reperfusion. **Abbreviations:** CF: Coronary Flow; AO: Aortic Output; Min: Minutes; PM: Perfusion Mode; WH: Working Heart.

Following global ischaemia, the heart was reperfused for 1 hour (10 minutes retrograded perfusion + 20 minutes Working Heart + 30 minutes retrograded perfusion). During each time period, haemodynamic data (**Section 2.2.15.5**) were recorded and the functional recovery calculated.

### 2.2.15.3. Regional Ischaemia-Reperfusion

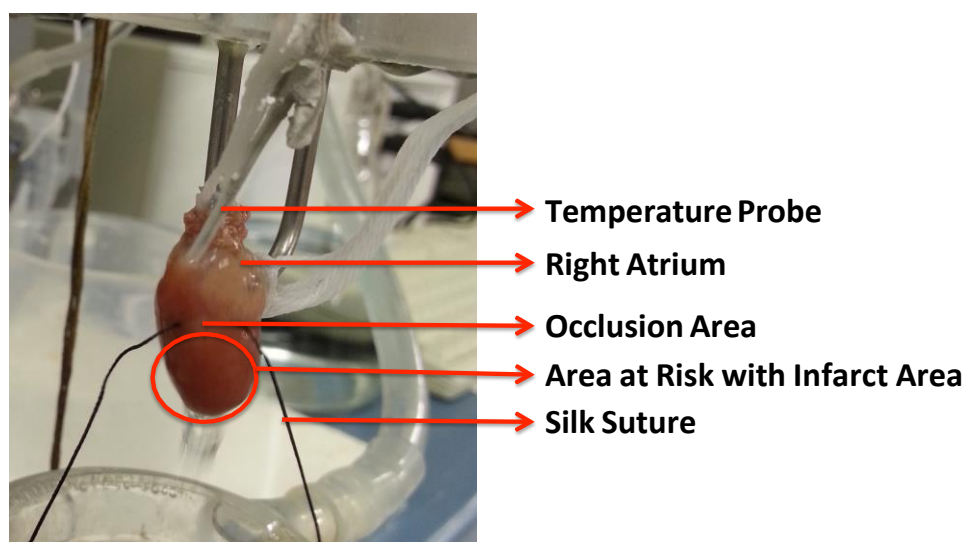
After the period of stabilization (30 minutes), hearts allocated for regional ischaemia-reperfusion studies were subjected to a regional ischaemia injury protocol (**Fig. 2.5**) by ligating the left anterior descending coronary artery of the heart. This was achieved by inserting a silk suture underneath and around the proximal left anterior descending coronary artery and tightening the ligation in a snare (**Fig. 2.6**).





**Fig. 2.5.** Protocol for Regional Ischaemia-Reperfusion. **Abbreviations:** CF: Coronary Flow; AO: Aortic Output; Min: Minutes; PM: Perfusion Mode; WH: Working Heart; LCA: Left Coronary Artery.

After the initial 30 minutes stabilization (as for global ischaemia; **Section 2.2.15.1**), the isolated heart perfusion system was switched back to retrograded perfusion mode before commencing with the regional ischaemia protocol.



**Fig. 2.6.** The Heart After Regional Ischaemia was Completed.

The snare was tightened until the CF was reduced to about 33 % and secured into place. Regional ischaemia injury was induced for 35 minutes at 36.8 °C. For the rat heart, optimum period of regional ischaemic injury has been determined according to literature to

be between 30 minutes and 40 minutes (Bell *et al.*, 2011). After 35 minutes of regional ischaemia, the snare was loosened and 1-hour reperfusion initiated (as for global ischaemia).

During each time period, haemodynamic data were recorded and the functional recovery and other parameters calculated (**Section 2.2.15.5**).

#### **2.2.15.4. Infarct Size Determination**

At the end of the regional ischaemia protocol, the silk suture that occluded the coronary artery was securely tied in a knot. Evans blue suspension (0,002 g / ml) was infused (Approximately 0.6 ml – 1 ml) via the aorta cannula until the correct coloring was observed. The heart was removed from the isolated heart perfusion system and stored at -10 °C for subsequent infarct size determination. Hearts were cut transversely into 2 mm segments and incubated for 30 minutes in the TTC containing buffer solution (20 ml of 100 mM  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ , 15.6 g / L distilled water (d. $\text{H}_2\text{O}$ ) added to 80 ml of 100 mM  $\text{Na}_2\text{HPO}_4$  14.2 g / L d. $\text{H}_2\text{O}$ ; 1 % w / v; pH 7.4 and TTC (0.05 g / heart) to generate a contrast in colouring between necrotic-, at-risk-, and viable tissue areas. The TTC solution was extracted after 1 hour and the slices of heart tissue fixed in formaldehyde solution (10 % v / v; 5 ml / heart) to halt the colouration process.

The heart tissue slices were removed from the formaldehyde solution, compressed between two glass plates, and scanned to produce a digital image. The digital image was used to determine the size of the left ventricle area at risk (AR; red colored tissue), the area of the necrotic infarcted tissue area (unstained, white colored tissue) in the risk zone, and the viable tissue area (VA; blue) by means of computerized planimetry (Image Tool: University of Texas: Health Science Center at San Antonio, Texas) (**Fig. 2.7**). Finally, the infarct size was expressed as a % of the AR.



**Fig. 2.7.** Representative example of a Heart Tissue Segment. **Colouration:** Infarct Area (IS): White; Area at Risk (AR): Red; Viable Area (VA): Blue.

#### 2.2.15.5. Haemodynamic Data for Isolated Heart Perfusions

Functional hemodynamic data was recorded both manually and by a computerized system (**Fig. 2.4 and Fig. 2.6**) that was connected via a pressure transducer to the isolated heart perfusion system. Manual measurements taken included the CF rate ( $< 20 \text{ ml / min}$ ) measured each time before the perfusion mode was changed and the aortic flow rate (Aortic output; AO;  $> 34 \text{ ml / min}$ ) measured at the end of each working heart mode. Total cardiac output (CO;  $\text{ml / min}$ ) was calculated as the sum of the CF and the AO.

The computerized system, connected to a pressure transducer (Viggo Spectromed) through a side branch of the aortic cannula, was used to record and calculate the intra-aortic pressure changes (Peak aortic systolic- and diastolic pressure; mmHg), the heart rate (HR) and the stroke volume (SV) ( $(\text{ml / min}) = \text{CO} / \text{HR}$ ).

Furthermore, the pressure power ( $W_p$ ) and kinetic power ( $W_k$ ) generated by the heart muscle were calculated to determine the total work power ( $W_t$ ) performance generated by the heart ( $W_t = W_p + W_k$ ). The computerized system also calculated the mean external power produced by the left ventricle (TW; mWatts) as  $\text{TW} = 0.002222 (\text{PAO} - 11.25)(\text{CO})$  where PAO = aortic pressure. Finally, the maximum rate of the left ventricular pressure rise ( $dP/dT_{\text{max}}$ ) and fall ( $dP/dT_{\text{min}}$ ) were also recorded by the computerized system. To determine functional recovery after ischaemic injury, post-ischaemic AO was expressed as a % of pre-ischaemic AO.

## 2.2.16. Western Blot Analysis

### 2.2.16.1. Proteins of Interest

*The following proteins of interest were analyzed:*

Total- and phosphorylated:

- eNOS (140 kDa) - Endothelial Nitric Oxide Synthase;
- PKB/Akt (58 kDa) - Protein Kinase B;
- AMPK (62 kDa) - 5' Adenosine Monophosphate-activated Protein Kinase.

Total:

- Nitrotyrosine (90 kDa);
- p22 Phox (22 kDa) - NADPH Oxidase;
- I $\kappa$ B $\alpha$  (55 kDa) - Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B-cells Inhibitor, Alpha.

### 2.2.16.2. Protein Extraction

Frozen cardiac tissue (approximately 30 mg / heart), previously stored in liquid nitrogen, was mechanically pulverized and fully homogenized (Polytron PT10 homogenizer; 2 x 5 seconds) in 800  $\mu$ l lysis buffer (**Table 2.2**) on ice. The supernatant containing the whole protein content of the samples was obtained by homogenizing the pulverized tissue samples in lysis buffer and centrifugation at 15 000 revolutions per minute (RPM's) for 15 minutes.

**Table 2.2.** *Preparation of Lysis Buffer.* **Abbreviations:** d.H<sub>2</sub>O: Distilled Water; EDTA: Ethylenediaminetetraacetic Acid; SDS: Sodium Dodecyl Sulphate; PMSF: Phenylmethylsulfonyl fluoride.

Reagent	Stock Concentration	Amount for 30ml
d.H <sub>2</sub> O		18.45 ml
Tris1HCl (pH 7.5)	20 nM	3.0 ml
EDTA	1 nM	300 µl
NaCl	150 nM	4.5 ml
β1glycerophosphate	1 nM	0,006 g
Sodiumpyrophosphate	2.5 nM	0,03 g
SDS	0.1 %	300 µl
Na <sub>3</sub> VO <sub>4</sub>	1 nM	300 µl
Triton® X1100	1 %	3 ml
LeupepQn	10 µg/ml	30 µl
AproQnin	10 µg/ml	30 µl
NaF	50 nM	0,0639 g
PMSF	50 µg/ml	90 µl – Added last

### 2.2.16.3. Determination of Protein Sample Concentrations

The protein concentration of the samples was determined by the Bradford method (Bradford, 1976). Diluted BSA solution (1:5; 100 µl BSA in 400 µl d.H<sub>2</sub>O) was used to prepare protein standards (total volume = 100 µl) in duplicate (0 µl, 5 µl, 10 µl, 20 µl, 40 µl, 80 µl BSA solution in 100 µl, 95 µl, 90 µl, 80 µl, 60 µl, 40 µl, and 20 µl d.H<sub>2</sub>O respectively). Duplicate samples of supernatant were diluted at a 1:10 and assayed at a 1:20 ratio. A volume of 900 µl Bradford reagent (500 mg commassie brilliant blue, 250 ml 95 % ethanol, 500 ml phosphoric acid; 1:5 dilution; 20 ml Bradford reagent in 80 ml d.H<sub>2</sub>O) was filtered (X 2) and added to each duplicate protein standard and samples and left for 15 minutes to allow the colour development to take place. Spectrophotometry (optical density = 595 nm) was used to determine the respective absorbance values and a standard curve was plotted (Absorbance vs. protein concentration).

#### 2.2.16.4. Preparation of the Lysates

Lysates were prepared by adding the respective amounts of the protein sample (determined by Bradford assay), sample buffer (2.5 mM Tris-HCl (pH 6.8); 25 % glycerol, 2 % SDS, 0.01 % bromophenol blue) and lysis buffer, boiled for 5 minutes (to allow for denaturation of proteins) and stored at -80 °C until further use.

#### 2.2.16.5. Protein Loading and Separation

The previously stored (-80 °C) lysates were boiled again for 5 minutes, centrifuged and loaded onto a polyacrylamide gel. Proteins were separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; 12 % loading gel: PKB/Akt, I $\kappa$ B $\alpha$ , p22 Phox, and AMPK; 7.5 % gel: eNOS, Nitrotyrosine) (**Table 2.3**).

**Table 2.3.** *Preparation of the Loading Gel.* **Abbreviations:** d.H<sub>2</sub>O: Distilled Water; SDS: Sodium Dodecyl Sulphate; APS: Ammonium Persulfate; TEMED: 1,2-Bis(dimethylamino)ethane.

Reagent	Stock Concentra.on	7.5 % Running Gel	12 % Running Gel
d.H <sub>2</sub> O (Millipore)		5.525 ml	4.4 ml
Tris5HCL (pH 8.8)	1.5 M	2.5 ml	2.5 ml
SDS	10 %	100 $\mu$ l	100 $\mu$ l
Acrylamide	40 %	1.875 ml	3.0 ml
APS	10 %	50 $\mu$ l	50 $\mu$ l
TEMED	99 %	20 $\mu$ l	20 $\mu$ l

The samples were subsequently loaded into wells created in the stacking gel (4% gel loaded on top of the loading gel) (**Table 2.4**).

**Table 2.4. Preparation of the Stacking Gel. Abbreviations:** d.H<sub>2</sub>O: Distilled Water; SDS: Sodium Dodecyl Sulphate; APS: Ammonium Persulfate; TEMED: 1,2-Bis(dimethylamino)ethane.

Reagent	Stock Concentration	4 % Casting Gel
d.H <sub>2</sub> O (Millipore)		3.05 ml
Tris-HCl (pH 6.8)	0.5 M	1.25 ml
SDS	10 %	50 µl
Acrylamide	40 %	500 µl
APS	10 %	50 µl
TEMED	99 %	10 µl

A molecular marker was loaded into the first well to locate the position of each protein after protein separation. A volume of 12 µl of each lysate sample (30 µg of protein) was loaded into each well consecutively and proteins separated according to their molecular weights during further electrophoresis in a running buffer (250 mM Tris, 192 mM glycine, and 1 % SDS) added to the protein separation system.

#### 2.2.16.6. Protein Transfer

The separated proteins were transferred onto PVDF membrane in transfer buffer (25 mM Tris-HCl, 192 mM glycine, and methanol (20 % v / v)) for 1 hour. The Ponceau staining technique was used to visually assess proper protein transfer and equal loading. The Ponceau red stain was washed off with TBS-tween (Tris-buffered saline and 0.1 % Tween<sup>®</sup> 20) before visualizing.

#### 2.2.16.7. Immunoblotting

Fat free powdered milk (5 %) dissolved in TBS-tween was used to block non-specific binding sites on all the membranes. After the membranes were thoroughly washed with TBS-Tween, they were incubated overnight at 4 °C in a polyclonal primary antibody solution (a 1:1000 dilution in TBS-Tween for all antibodies), specific to each phosphorylated- and/or total protein. Following the overnight incubation, the membranes

were thoroughly washed in TBS-Tween to remove non-immobilized primary antibody. Immobilized primary antibody was then conjugated with secondary antibody (Horseradish peroxidase-conjugated anti-rabbit) diluted in TBS-Tween (1:4000 dilution) for 1 hour at room temperature, followed by another thorough wash with TBS-Tween.

#### **2.2.16.8. Protein Detection and Quantification**

The proteins were detected by covering the membranes with ECL detection reagent. In the first set of Western blot analysis the light emitting from the luminescence was captured on medical x-ray film and the image analysed by densitometry. Where necessary,  $\beta$ -tubulin was used as a control for equal protein loading. The Western blot analyses of hearts after a global ischaemic insult were performed by utilizing the Chemidoc<sup>™</sup> MP Imager System with Image Lab<sup>™</sup> - 5 software. Values were obtained and normalized to the corresponding controls for equal protein loading.

### **2.3. Statistical Analysis**

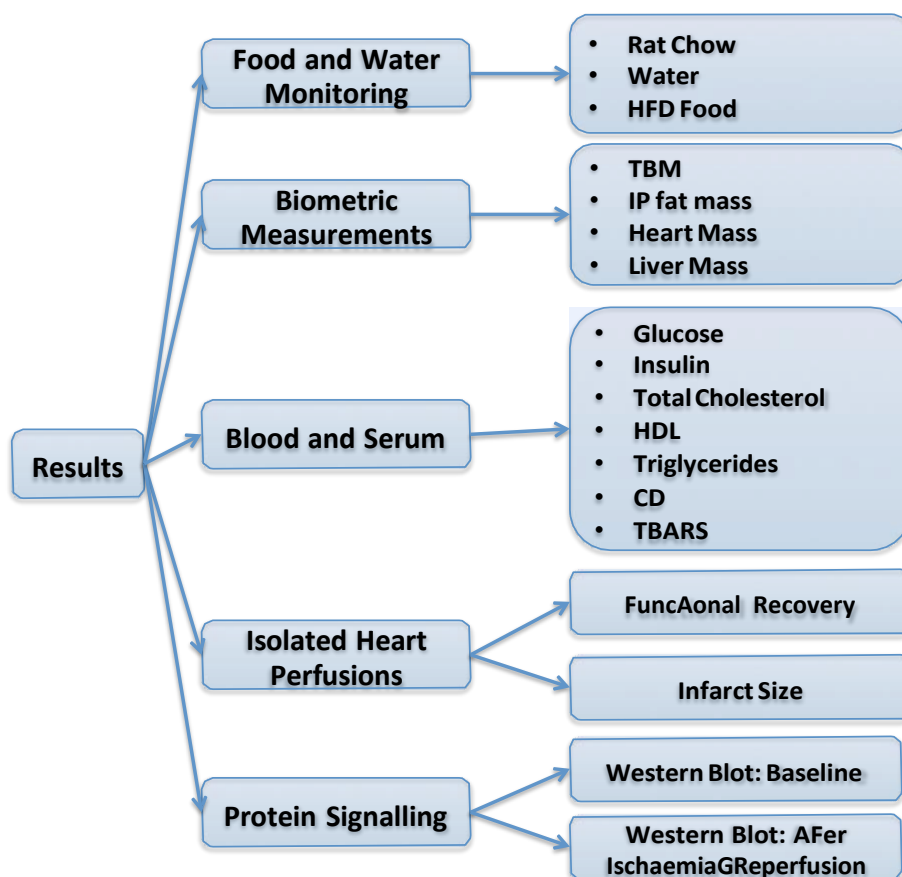
GraphPad Prism<sup>®</sup> (version 6) was used for all statistical analysis. A student's t-test was used for comparison of two variables. A one- or two-way analysis of variance (ANOVA) with Bonferroni post hoc correction was used for more the comparison of more than two variables. Values were expressed as the mean  $\pm$  standard error of the mean (SEM). Statistical significance was set at a p-value < 0.05.



## Chapter 3 – Results

### 3.1. Introduction

This chapter describes the data from the food and water monitoring, biometric measurements (TBM, IP fat mass, heart mass, liver mass), serum biochemical analyses from fasted animals (glucose, insulin, TC, HDL cholesterol, TG, CD, and TBARS levels), isolated heart perfusion experiments (functional recovery and infarct size) and protein signalling in cardiac tissue: Western blot analysis of important signalling proteins in the NOS, ROS and inflammatory protein signalling cascade (baseline and after ischaemia-reperfusion) (**Fig. 3.1**).

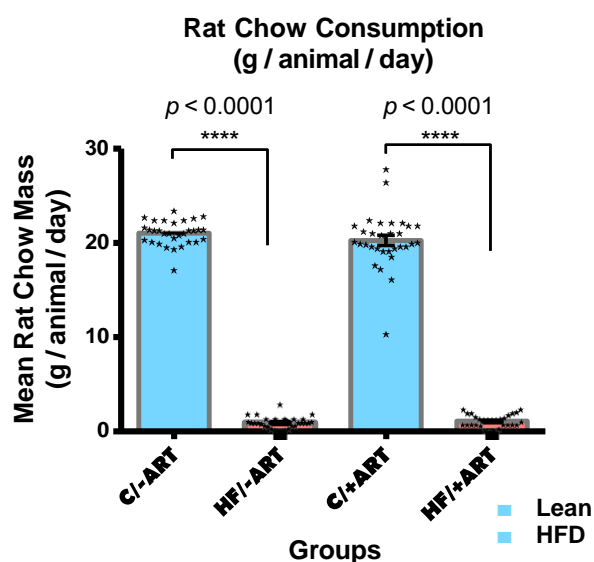


**Fig. 3.1.** Chapter 3: Results Outline. **Abbreviations:** TBM: Total Body Mass; IP: Intra-Peritoneal; HDL: High Density Lipoproteins.

## 3.2. Food and Water Monitoring During the First Month of the Drug Treatment Programme

### 3.2.1. Rat Chow Consumption

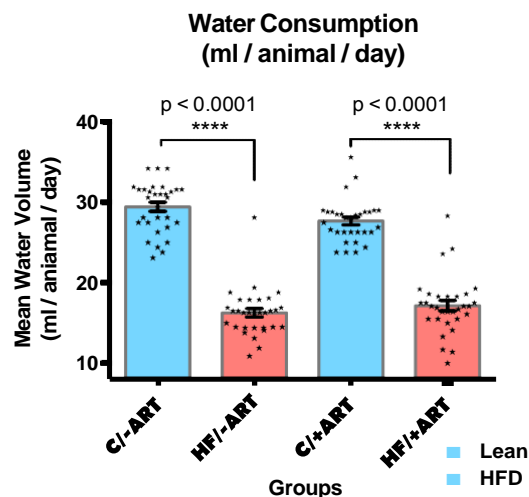
The normal rat chow consumption (g / animal / day) remained constant for all the groups during the first 31 days of drug administration. The untreated and treated lean, control groups consumed more rat chow compared to their respective HFD counterparts. There were no differences between the untreated vs. treated lean, control and HFD groups respectively (**Fig. 3.2**; See **Appendix D** for Food and Water Monitoring).



**Fig. 3.2.** Mean Rat Chow Consumption / Animal (g / animal / day) (data expressed as mean  $\pm$  SEM / group). CI-ART:  $21.05 \pm 0.23$  g / animal / day; HF/-ART:  $0.95 \pm 0.11$  g / animal / day; CI/+ART:  $20.27 \pm 0.53$  g / animal / day; HF/+ART:  $1.10 \pm 0.11$  g / animal / day. N = 20 / group.

### 3.2.2. Water Consumption

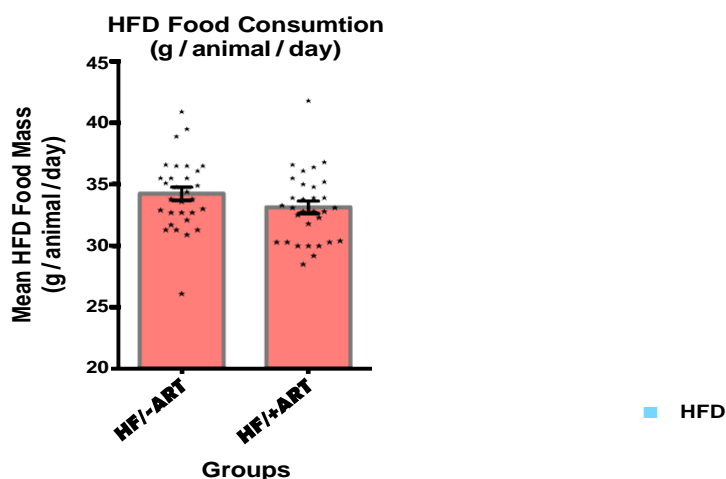
The water consumption (ml / animal / day) also remained constant for all the groups during the first 31 days of drug administration. The untreated and treated lean, control groups consumed more water on a daily basis compared to their respective HFD counterparts. There were no differences between the untreated vs. treated lean, control and HFD groups respectively (**Fig. 3.3**).



**Fig. 3.3.** Mean Water Consumption / Animal (ml / animal / day) (data expressed as mean  $\pm$  SEM / group). C/-ART: 29.43  $\pm$  0.56 ml / animal / day; HF/-ART: 16.25  $\pm$  0.53 ml / animal / day; C/+ART: 27.66  $\pm$  0.47 ml / animal / day and HF/+ART: 17.14  $\pm$  0.65 ml / animal / day. N = 20/ group.

### 3.2.3. High Fat Diet (HFD) Consumption

The HFD groups (HF/-ART and HF/+ART) consumed the same amount (g / animal / day) of HFD food per animal daily, during the first 31 days of the drug treatment programme (**Fig. 3.4**).



**Fig. 3.4.** Mean High Fat Food Consumption / Animal (g/animal/day) (data expressed as mean  $\pm$  SEM / group). HF/-ART: 34.24  $\pm$  0.53 g / animal / day and HF/+ART: 33.14  $\pm$  0.51 g / animal / day. N = 20 / group.

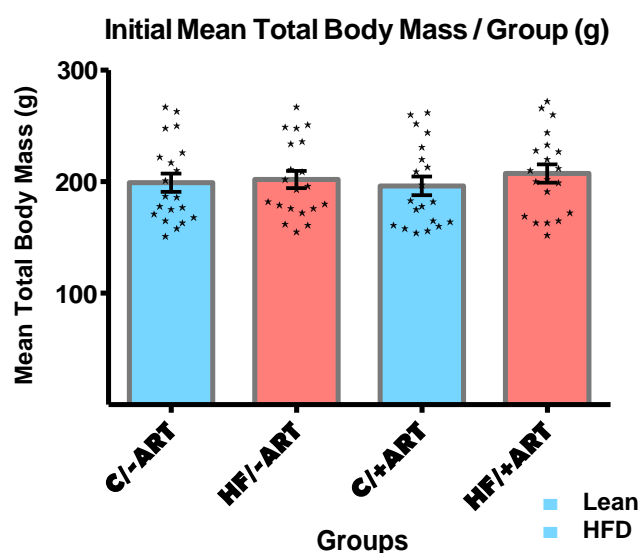
### 3.3. Biometric Measurements During and After the Feeding and Drug Treatment Programme

Biometric measurements (TBM, IP fat mass, heart mass and liver mass) were recorded during the feeding and drug treatment programme and on the day the animals were sacrificed.

#### 3.3.1. Total Body Mass (TBM)

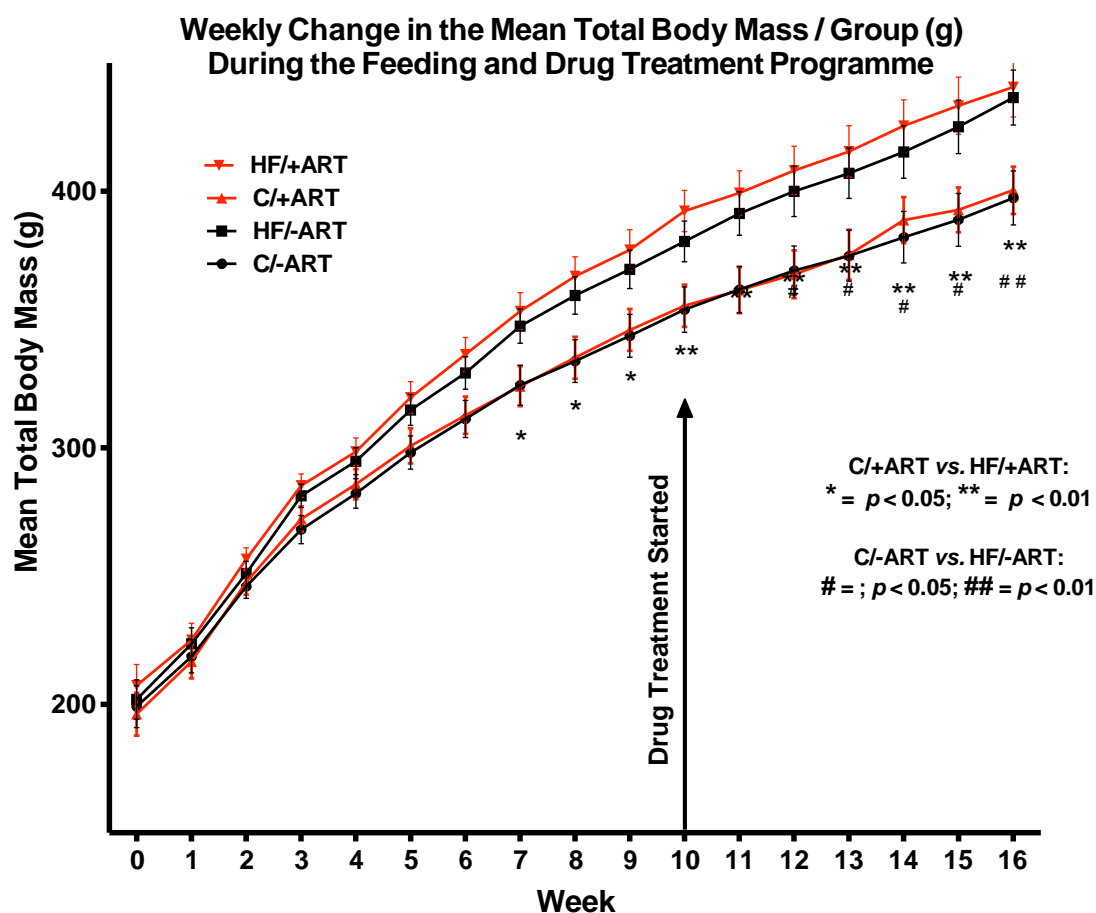
##### 3.3.1.1. Changes in Total Body Mass (TBM) During the Feeding and Drug Treatment Programme

The TBM (g) of the animals of all groups at the commencement of the feeding and drug treatment programme ranged between 151 g and 272 g. There were no differences in the initial mean TBM (g) / group (C/-ART, HF/-ART, C/+ART, HF/+ART) at the start of the feeding programme (**Fig. 3.5**).



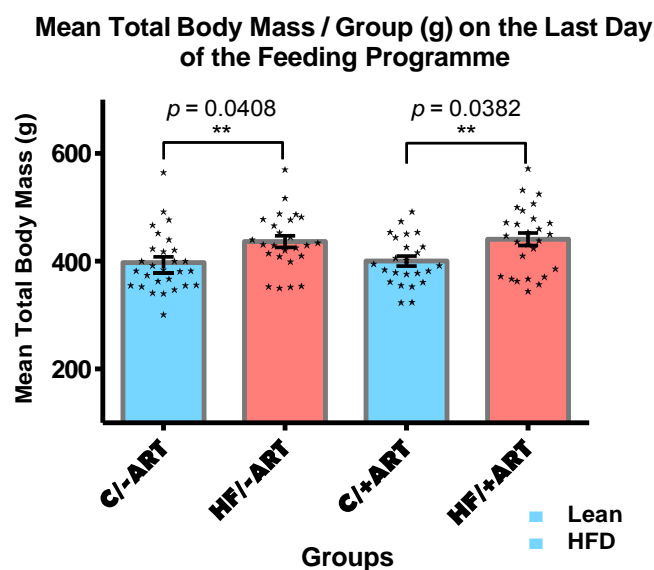
**Fig. 3.5.** Mean TBM / Group (g on Day 1 of the Feeding and Drug Treatment Programme (data expressed as mean TBM ± SEM / group). C/-ART: 199.20 ± 8.18 g; HF/-ART: 202.00 ± 7.70 g; C/+ART: 196.20 ± 8.36 g; HF/+ART: 207.40 ± 8.16 g. N = 20 / group.

The mean TBM of all groups increased during the 16-week feeding and drug treatment programme. The C/-ART group had a 99.50 % higher final mean TBM compared to the initial mean TBM; The HF/-ART group had a 116.14 % higher mean TBM compared to the initial mean TBM; The C/+ART group had a 104.08 % higher final mean TBM compared to initial mean TBM and the HF/+ART had a 112.49 % higher mean TBM compared to the initial mean TBM (**Fig. 3.6**).



**Fig. 3.6.** Weekly Change in the Mean TBM / Group (g) (data expressed as mean TBM  $\pm$  SEM / group,  $p < 0.05$ ).

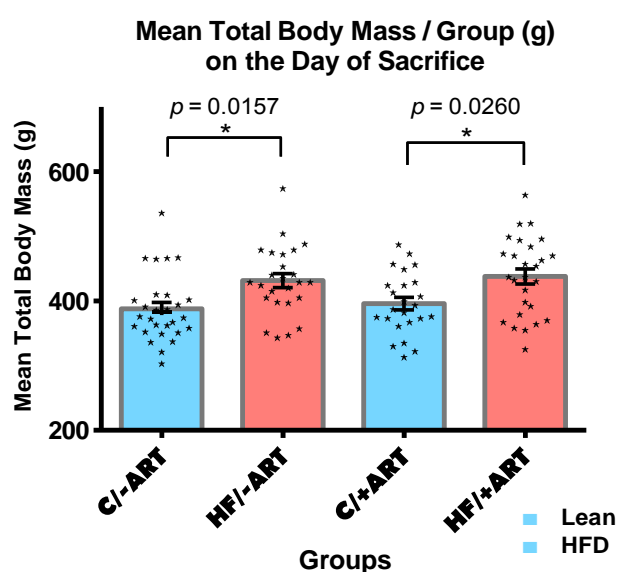
At the end of the 16-week feeding and drug treatment programme, the mean TBM of the HF/-ART and HF/+ART groups was 39.13 g, and 40.25 g higher compared to their respective age-matched lean, control groups (vs. C/-ART: 9.86 %;  $p = 0.0408$  and vs. C/+ART: 10.06 %;  $p = 0.0382$ ). The ART drug treatment *per se* had no additional effects on the mean TBM in the lean, control and HFD groups compared to their respective untreated control groups (C/-ART vs. C/+ART; and HFD/-ART vs. HF/+ART) (**Fig. 3.7**).



**Fig. 3.7.** Mean TBM / Group (g) on the Last Day of the Feeding and Drug Treatment Programme (data expressed as mean TBM  $\pm$  SEM / group). C/-ART: 397.40  $\pm$  10.53 g; HF/-ART: 436.60  $\pm$  10.68 g; C/+ART: 400.40  $\pm$  9.24 g and HF/+ART: 440.70  $\pm$  11.66 g. N = 25 – 28 / group.

### 3.3.1.2. Mean Total Body Mass (TBM) / Group on the Day of Sacrifice

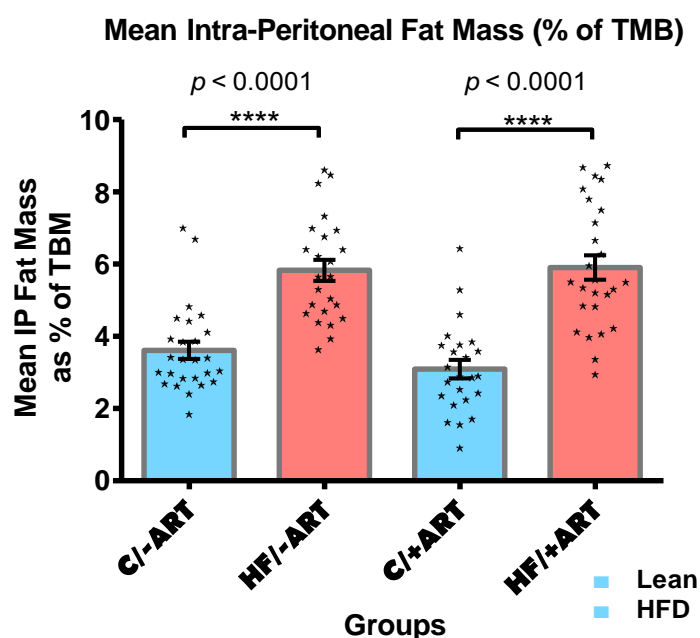
On the day of sacrifice, the mean TBM (g) was 43.63 g higher in the HF/-ART compared to the C/-ART group (11.25 %;  $p = 0.0157$ ), and 41.88 g higher in the HF/+ART compared to the C/+ART group (9.56 %;  $p = 0.026$ ). There were no differences between the untreated lean, control (C/-ART vs. C/+ART) and HFD (HFD/-ART vs. HFD/+ART) groups respectively (**Fig. 3.8**).



**Fig. 3.8.** Mean TBM / Group (g) on the Day of Sacrifice (data expressed as mean  $\pm$  SEM / group). C/-ART (N = 28): 387.90  $\pm$  9.81 g; HF/-ART (N = 25): 431.60  $\pm$  10.70 g; C/+ART (N = 24): 396.00  $\pm$  9.72 g and HF/+ART (N = 27): 437.90  $\pm$  11.63 g. N = 24 – 28 / group.

### 3.3.2. Intra-Peritoneal (IP) Fat Mass

The mean IP fat mass / group (expressed as a % of TBM) was 61.24 % higher in the HF/-ART group compared to the C/-ART group ( $p < 0.0001$ ), and 90.98 % higher in the HF/+ART compared to the C/+ART group ( $p < 0.0001$ ). There were no differences between lean, control (C/-ART vs. C/+ART) and the HFD (HF/-ART vs. HF/+ART) groups (**Fig. 3.9**).

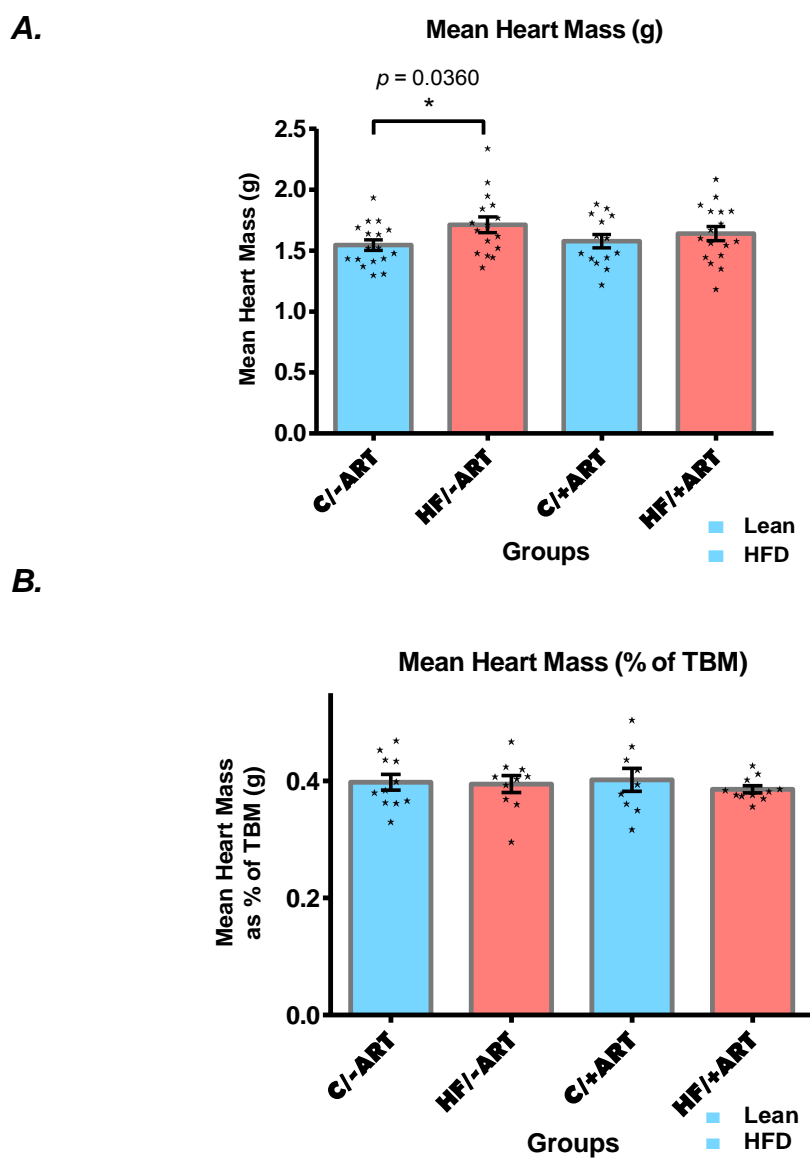


**Fig. 3.9.** Mean IP Fat Mass / Group (% of TBM) (data expressed as mean  $\pm$  SEM / group). C/-ART:  $3.61 \pm 0.24$  %; HF/-ART:  $5.82 \pm 0.29$  %; C/+ART:  $3.09 \pm 0.25$  % and HF/+ART:  $5.91 \pm 0.34$  %. N = 24 – 26 / group.



### 3.3.3. Heart Mass

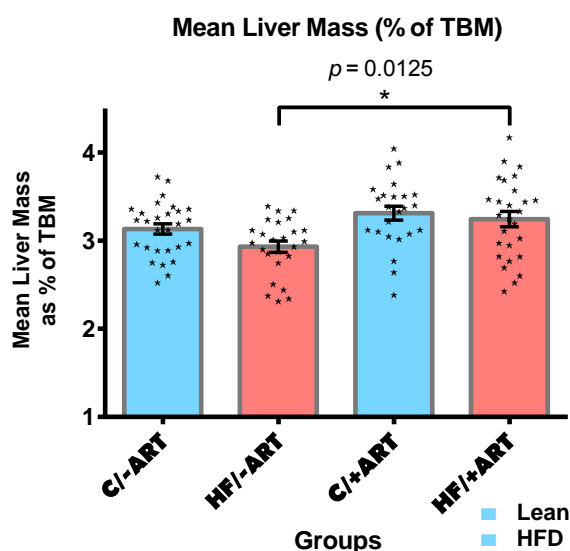
The actual mean heart mass (g) of the HF/-ART group was 0.17 g higher compared to the C/-ART group (10.32 %;  $p < 0.0360$ ) (**Fig. 3.10 A**). The mean heart mass / group (expressed as % of TBM) showed no differences between any of the groups (**Fig. 3.10 B**). No other differences between any groups were observed.



**Fig. 3.10.** Heart Mass / Group (% of TBM (**A**) and g (**B**)) (data expressed as mean  $\pm$  SEM / group). % of TBM: C/-ART:  $0.40 \pm 0.01$  %; HF/-ART:  $0.39 \pm 0.01$  %; C/+ART:  $0.40 \pm 0.019$  % and HF/+ART:  $0.39 \pm 0.01$  %. Heart mass in g: C/-ART:  $1.55 \pm 0.04$  g; HF/-ART:  $1.71 \pm 0.06$  g; C/+ART:  $1.58 \pm 0.06$  g and HF/+ART:  $1.64 \pm 0.06$  g. N = 9 – 11 / group.

### 3.3.4. Liver Mass

The mean liver mass / group (expressed as a % of TBM) was significantly higher in the HF/+ART group vs. HF/-ART: 0.32 % (10.92 % increase;  $p = 0.0125$ ). No further differences were observed in any of the groups (**Fig. 3.11**). There were also no differences in the actual liver mass / group (g) between any of the groups.

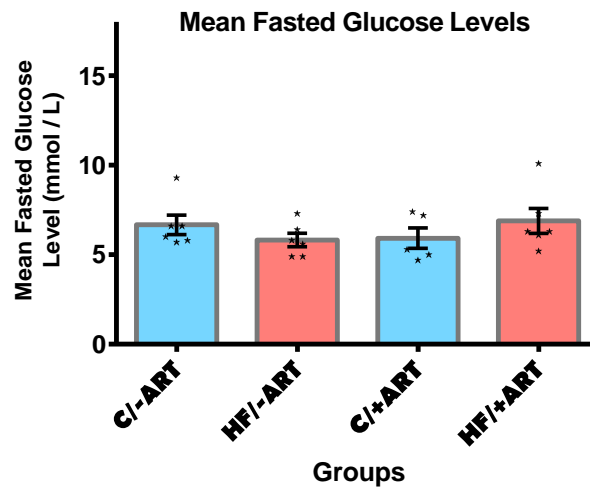


**Fig. 3.11.** Mean Liver Mass / Group (% of TBM) (data expressed as mean  $\pm$  SEM / group). C/-ART: 3.13  $\pm$  0.58 %; HF/-ART: 2.93  $\pm$  0.64 %; C/+ART: 3.31  $\pm$  0.08 % and HF/+ART: 3.25  $\pm$  0.09 %. N = 24 – 28 / group.

### 3.4. Biochemical Analyses – Glucose, Insulin and Lipid Level Determination.

#### 3.4.1. Glucose Levels

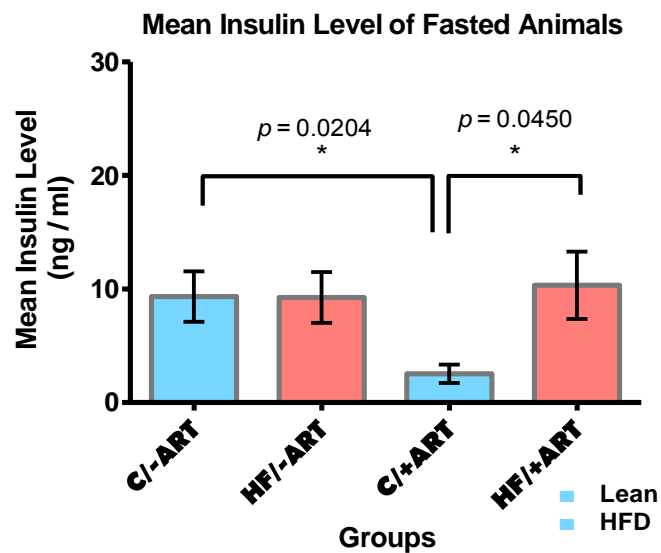
There were no differences in the mean glucose levels / group between any of the groups. (Fig. 3.12).



**Fig. 3.12.** Mean Fasted Glucose Levels / Group (mmol / L) (data expressed as mean  $\pm$  SEM / group). C/-ART: 6.67  $\pm$  0.55 mmol / L; HF/-ART: 5.82  $\pm$  0.38 mmol / L; C/+ART: 8.92  $\pm$  0.57 mmol / L and HF/+ART: 6.88  $\pm$  0.70 mmol / L. N = 5 – 6 / group.

### 3.4.2. Insulin Levels

Mean fasting insulin levels were significantly lower in the C/+ART group compared to other groups (vs. C-ART: 270.63 %;  $p = 0.0204$ ; vs. HF/+ART: 359.11 %;  $p = 0.045$ ) (**Fig. 3.13**).

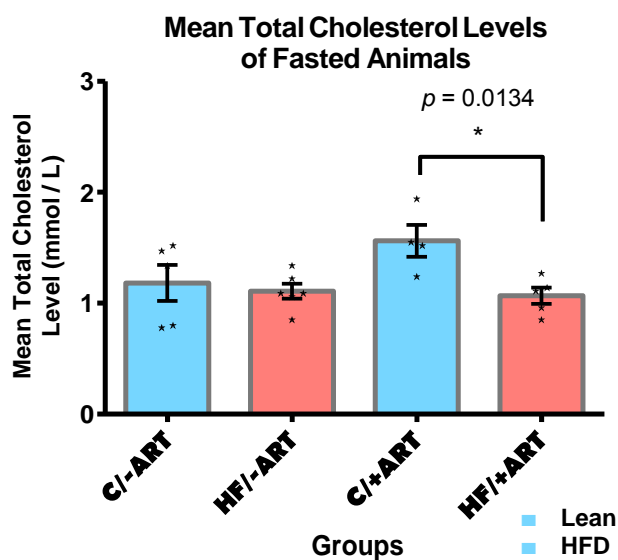


**Fig. 3.13.** Mean The Insulin Levels / Group (ng / ml) (data expressed as mean  $\pm$  SEM / group). C/-ART:  $9.34 \pm 2.22$  ng / ml; HF/-ART:  $9.26 \pm 2.24$  ng / ml; C/+ART:  $2.52 \pm 0.80$  ng / ml and HF/+ART:  $10.33 \pm 2.96$  ng / ml. N = 5 – 6 / grou

### 3.4.3. Lipid Profile

#### 3.4.3.1. Total Cholesterol (TC) Levels

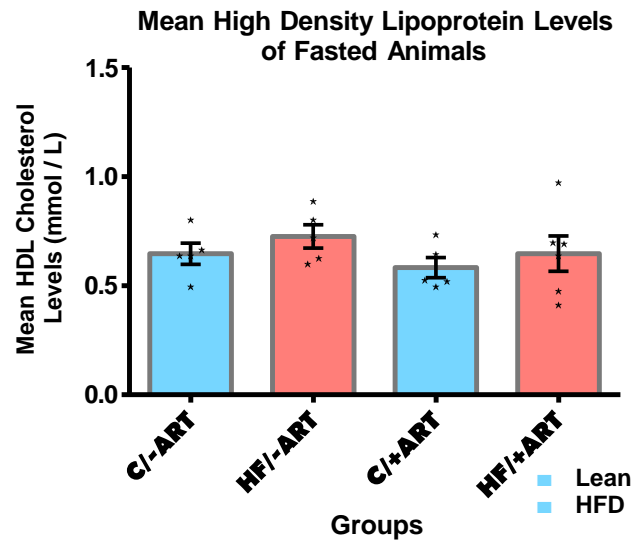
Mean fasting TC levels / group (mmol / L) were 45.79 % lower in the HF/+ART compared to C/+ART ( $p = 0.0134$ ). No further differences were observed between any of the groups (**Fig. 3.14**).



**Fig. 3.14.** Mean Total Cholesterol Levels / Group (mmol / L) (data expressed as mean  $\pm$  SEM / group). C/-ART:  $1.18 \pm 0.16$  mmol / L; HF/-ART:  $1.11 \pm 0.067$  mmol / L; C/+ART:  $1.56 \pm 0.14$  mmol / L and HF/+ART:  $1.07 \pm 0.07$  mmol / L. N = 4 – 6 / group.

### 3.4.3.2. High Density Lipoprotein (HDL) Cholesterol Levels

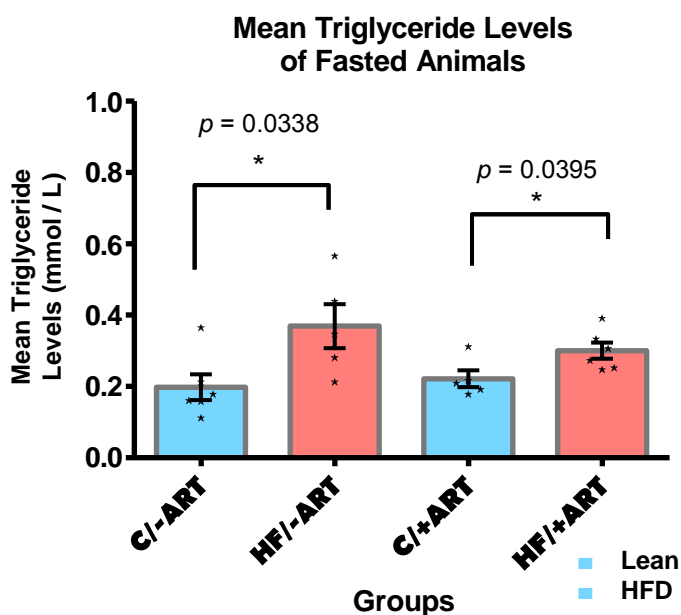
The mean HDL cholesterol levels (mmol / L) did not differ between any of the groups (**Fig. 3.15**).



**Fig. 3.15.** Mean HDL Cholesterol Levels / Group (mmol / L) (data expressed as mean  $\pm$  SEM / group). C/-ART (N = 5):  $0.65 \pm 0.049$  mmol / L; HF/-ART (N = 5):  $0.73 \pm 0.05$  mmol / L; C/+ART (N = 5):  $0.58 \pm 0.05$  mmol / L and HF/+ART (N = 6):  $0.65 \pm 0.08$  mmol / L. N = 5 – 6 / group.

### 3.4.3.3. Triglyceride (TG) Levels

The mean TG levels / group were 85.00 % higher in the HF/-ART group compared to the C/-ART group ( $p = 0.0338$ ), and 36.36 % higher in the HF/+ART group compared to the C/+ART group ( $p = 0.0395$ ) (**Fig. 3.16**).



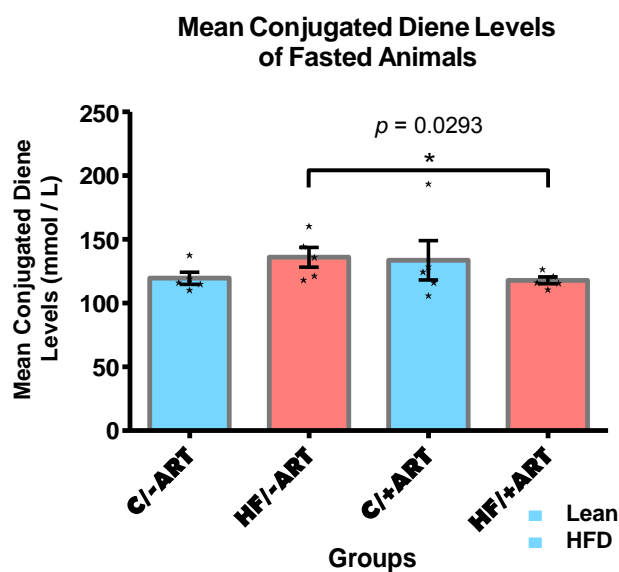
**Fig. 3.16.** Mean TG Levels / Group (mmol / L) (data expressed as mean  $\pm$  SEM / group). C/-ART (N = 4): 0.20  $\pm$  0.04 mmol / L; HF/-ART (N = 5): 0.37  $\pm$  0.061 mmol / L; C/+ART (N = 5): 0.22  $\pm$  0.02 mmol / L and HF/+ART (N = 6): 0.30  $\pm$  0.02 mmol / L. N = 5 – 6 / group.

### 3.4.4. Indicators of Lipid Peroxidation and Oxidative Stress

TBARS and CD levels were chosen to assess lipid peroxidation and oxidative stress as they have been implicated in the potential pathways, linking oxidation status to the pathophysiological pathways that leads to CVD. TBARS in particular have been linked to represent a composite number of oxidative damage products such as malondialdehyde and has also been associated with the progression of carotid atherosclerosis (Trevisan *et al.* 2001).

#### 3.4.4.1. Conjugated Diene (CD) Levels

The CD level was 18.1 mmol / L lower in the in the HF/+ART group compared tot the HF/-ART group ( $p = 0.0293$ ). There were no further differences observed in the mean CD levels (mmol / L) between any of the other groups (**Fig. 3.17**).

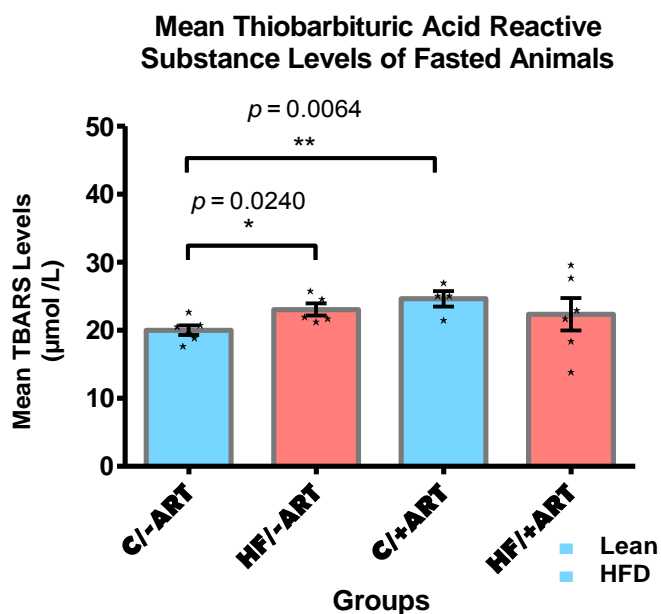


**Fig. 3.17.** Mean CD Levels / Group (mmol / L) (data expressed as mean  $\pm$  SEM / group). C/-ART: 119.50  $\pm$  4.74 mmol / L; HF/-ART: 136.00  $\pm$  7.76 mmol / L; C/+ART: 133.60  $\pm$  15.48 mmol / L and HF/+ART: 117.90  $\pm$  2.71 mmol / L. N = 5 – 6 / group.



### 3.4.4.2. Thiobarbituric Acid Reactive Substance (TBARS) Levels

The mean TBARS levels / group ( $\mu\text{mol} / \text{L}$ ) were 15.19 % higher in the HF/-ART group ( $p = 0.024$ ), and 23.09 % higher in the C/+ART group compared to the untreated, lean, control (C/-ART) group ( $p = 0.024$ ). No further differences were observed (**Fig. 3.18**).



**Fig. 3.18.** Mean TBARS Levels / Group ( $\mu\text{mol}/\text{L}$ ) (data expressed as mean  $\pm$  SEM / group). C/-ART:  $20.01 \pm 0.71 \mu\text{mol} / \text{L}$ ; HF/-ART:  $23.05 \pm 0.90 \mu\text{mol} / \text{L}$ ; C/+ART:  $24.63 \pm 1.15 \mu\text{mol} / \text{L}$  and HF/+ART:  $22.35 \pm 2.38 \mu\text{mol} / \text{L}$ . N = 4 – 6 / group.

### 3.5. Isolated Heart Perfusions

All hearts were perfused on the Langendorff retrograded perfusion system to determine functional recovery after a global ischaemia-reperfusion insult and infarct size after a regional ischaemia-reperfusion insult.

#### 3.5.1. Global Ischaemia-Reperfusion

##### 3.5.1.1. Functional Recovery after Global Ischaemia-Reperfusion

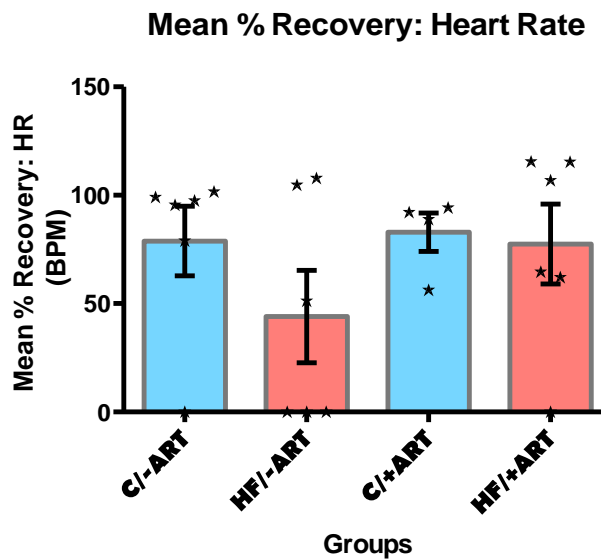
In order to assess functional recovery after a global ischaemia-reperfusion insult, six endpoints were measured before and after the global ischaemia-reperfusion insult, namely: heart rate (beats / min), CF (ml / min), AO (ml / min), total CO (ml / min), peak systolic pressure (PSP; mmHg) and total power (mW). These endpoints were measured after 30 minutes of stabilization (15 minutes of retrograde perfusion + 15 minutes of working heart perfusion) prior to ischaemia (pre-ischaemia-reperfusion; baseline) and at the end of the ischaemia-reperfusion protocol (post-ischaemia-reperfusion). Post-ischaemia-reperfusion values were compared to pre-ischaemia-reperfusion, baseline values to determine functional recovery and expressed as a percentage of the pre-ischaemia values (% recovery). As the pre-ischaemic protocol for both global and regional ischaemia-reperfusion was the same, these two sample groups were grouped together to yield a larger sample size. The results for global ischaemia-reperfusion are summarised in **Table 3.1** (See **Appendix D** for Other Haemodynamic Data for Global Ischaemia-Reperfusion).

**Table 3.1.** Summary of Haemodynamic Data – Global Ischaemia-Reperfusion (data expressed as mean  $\pm$  SEM / group (% Recovery)). **Abbreviations:** HR: Heart Rate; AO: Aortic Output; CF: Coronary Flow; CO: Total Cardiac Output; PSP: Peak Systolic Pressure; Wt: Total Power; Pre: Pre-ischaemia-reperfusion; Post: Post-Ischaemia-reperfusion. **Pre-Ischaemia vs. Post-Ischaemia:** \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ ; \*\*\*\* =  $p < 0.0001$ .

Haemodynamic Data					
Pre\$Ischaemia vs. Post\$Ischaemia					
Mean $\pm$ SEM (% Recovery)		C/:ART (Pre: N = 16; Post: N = 6)	HF/:ART (Pre: N = 14; Post: N = 6)	C/+ART (Pre: N = 11; Post: N = 4)	HF/+ART (Pre: N = 13; Post: N = 6)
HR (BPM)	Pre	224.80 $\pm$ 12.67	234.80 $\pm$ 8.72	245.90 $\pm$ 6.18	259.40 $\pm$ 17.47
	Post	205.80 $\pm$ 41.72	105.30 $\pm$ 50.13 ***	210.00 $\pm$ 23.82	201.50 $\pm$ 45.35
% Recovery		78.87 %	44.02 %	82.94 %	77.78 %
AO (ml / min)	Pre	42.09 $\pm$ 1.51	41.21 $\pm$ 1.48	42.36 $\pm$ 1.87	38.46 $\pm$ 1.57
	Post	16.58 $\pm$ 5.34 ****	7.08 $\pm$ 5.27 ****	14.50 $\pm$ 5.85 ****	7.16 $\pm$ 4.83 ****
% Recovery		39.34 %	16.99 %	32.12 %	19.16 %
CF (ml / min)	Pre	14.13 $\pm$ 0.74	14.11 $\pm$ 0.51	15.36 $\pm$ 0.74	16.08 $\pm$ 1.13
	Post	10.75 $\pm$ 2.17	6.25 $\pm$ 2.58 ***	15.75 $\pm$ 4.08	10.75 $\pm$ 2.40 *
% Recovery		76.48 %	44.12 %	93.86 %	68.16 %
CO (ml / min)	Pre	56.22 $\pm$ 1.84	55.32 $\pm$ 1.73	57.91 $\pm$ 1.81	54.54 $\pm$ 2.41
	Post	27.33 $\pm$ 7.00 ****	13.33 $\pm$ 7.56 ****	30.25 $\pm$ 9.84 ***	17.92 $\pm$ 6.10 ****
% Recovery		48.55 %	23.28 %	48.92 %	32.59 %
PSP (mmHG)	Pre	105.10 $\pm$ 1.57	100.80 $\pm$ 2.05	101.70 $\pm$ 1.69	99.15 $\pm$ 2.03
	Post	83.32 $\pm$ 16.79	41.00 $\pm$ 19.17 ****	86.0 $\pm$ 8.37	66.83 $\pm$ 16.79 *
% Recovery		77.80 %	40.94 %	82.94 %	64.44 %
Wt (mW)	Pre	13.24 $\pm$ 0.52	12.35 $\pm$ 0.43	13.24 $\pm$ 0.53	12.24 $\pm$ 0.61
	Post	6.08 $\pm$ 1.58 ****	2.44 $\pm$ 1.68 ****	6.37 $\pm$ 2.28 ***	3.56 $\pm$ 1.41 ****
% Recovery		44.90 %	19.45 %	44.21 %	27.07 %

### 3.5.1.1.1. Heart Rate (HR)

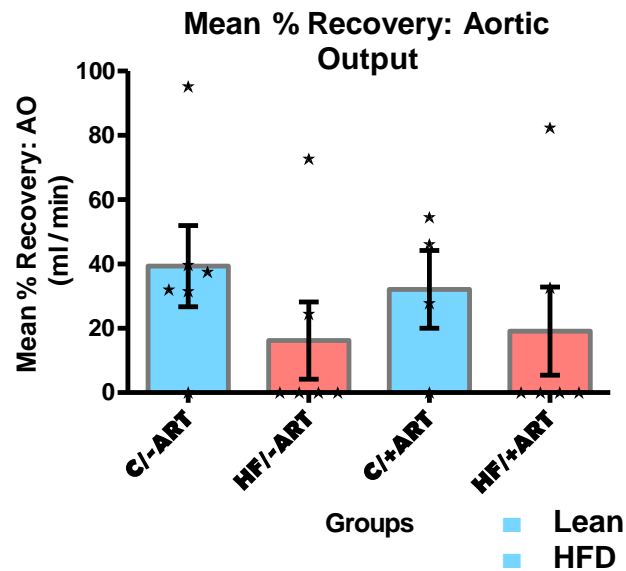
There were also no differences in the mean % recovery of HR between any of the groups (**Fig. 3.19**).



**Fig. 3.19.** Mean HR % Recovery After Global Ischaemia-Reperfusion (data expressed as mean  $\pm$  SEM / group). C/-ART:  $87.87 \pm 16.11$  %; HF/-ART:  $44.02 \pm 21.33$  %; C/+ART:  $82.94 \pm 8.93$  %; HF/+ART:  $77.48 \pm 18.40$  %. N = 4 – 6 / group.

### 3.5.1.1.2. Aortic Output (AO)

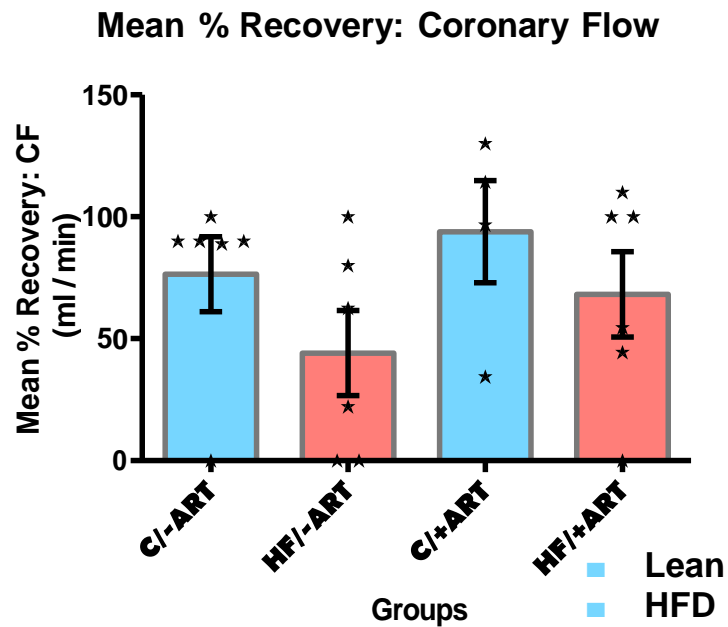
There were no differences in the mean % recovery of AO between any of the groups (**Fig. 3.20**).



**Fig. 3.20.** Mean OA % Recovery After Global Ischaemia-Reperfusion (data expressed as mean  $\pm$  SEM / group). C/-ART: 39.34  $\pm$  12.64 %; HF/-ART: 16.19  $\pm$  11.99 %; C/+ART: 32.12  $\pm$  12.08 %; HF/+ART: 19.16  $\pm$  13.71 %. N = 4 – 6 / group.

### 3.5.1.1.3. Coronary Flow (CF)

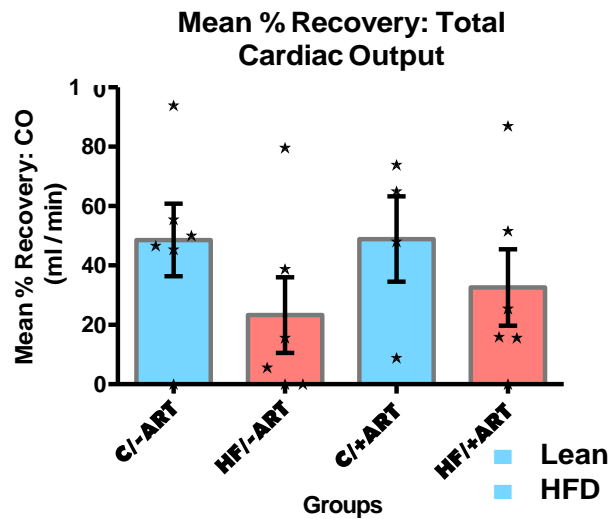
There were no differences in the mean % recovery of CF between any of the groups (**Fig. 3.21**).



**Fig. 3.21.** Mean CF % Recovery After Global Ischaemia-Reperfusion (data expressed as mean  $\pm$  SEM / group). C/-ART: 76.48  $\pm$  15.39 %; HF/-ART: 44.12  $\pm$  17.44 %; C/+ART: 93.86  $\pm$  20.93 %; HF/+ART: 68.16  $\pm$  17.48 %. N = 4 – 6 / group

### 3.5.1.1.4. Cardiac Output (CO)

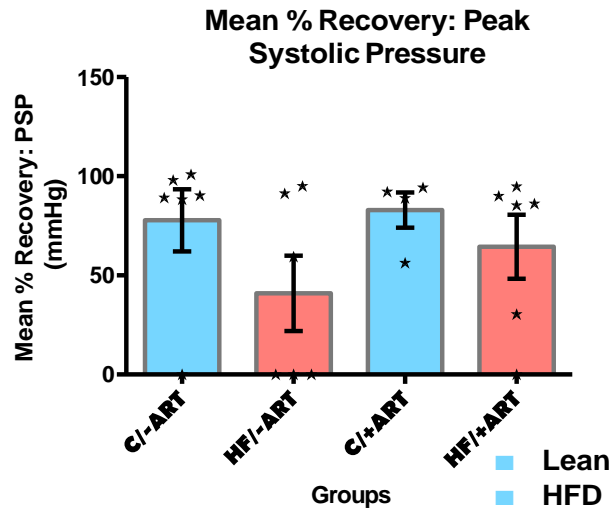
There were no differences in the mean % recovery of CO between any of the groups (**Fig. 3.22**).



**Fig. 3.22.** Mean CO % Recovery After Global Ischaemia-Reperfusion (data expressed as mean  $\pm$  SEM / group). C/-ART: 48.55  $\pm$  12.21 %; HF/-ART: 23.28  $\pm$  12.75 %; C/+ART: 48.92  $\pm$  14.39 %; HF/+ART: 32.59  $\pm$  12.91 %. N = 4 – 6 / group.

### 3.5.1.1.5. Peak Systolic Pressure (PSP)

There were no differences in the mean % recovery of PSP between any of the groups (**Fig. 3.23**).

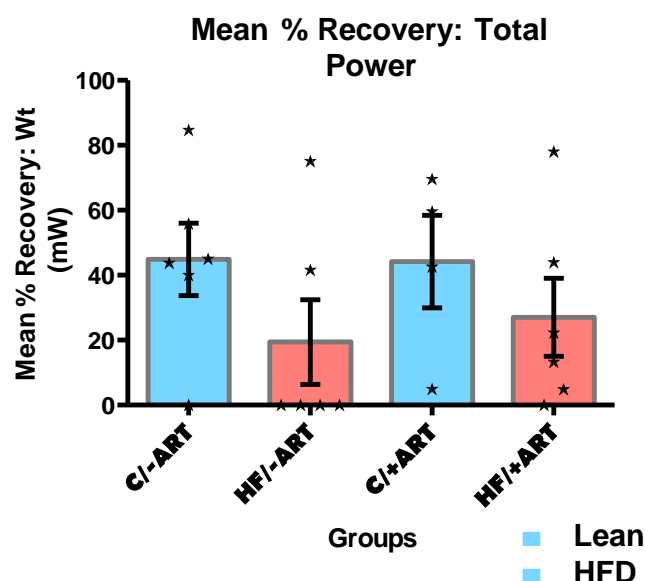


**Fig. 3.23.** Mean PSP % Recovery After Global Ischaemia-Reperfusion (data expressed as mean  $\pm$  SEM / group). CI-ART: 77.80  $\pm$  15.70 %; HF/-ART: 40.94  $\pm$  18.99 %; CI/+ART: 82.94  $\pm$  8.93 %; HF/+ART: 64.44  $\pm$  16.12 %. N = 4 – 6 / group.



### 3.5.1.1.6. Total Power (Wt)

There were no differences in the % recovery Wt between any of the groups (**Fig. 3.24**).



**Fig. 3.24.** Mean Wt % Recovery After Global Ischaemia-Reperfusion (data expressed as mean  $\pm$  SEM / group). C/-ART: 44.90  $\pm$  11.17 %; HF/-ART: 19.45  $\pm$  13.04 %; C/+ART: 44.21  $\pm$  14.22 %; HF/+ART: 27.07  $\pm$  12.00 %. N = 4 – 6 / group.

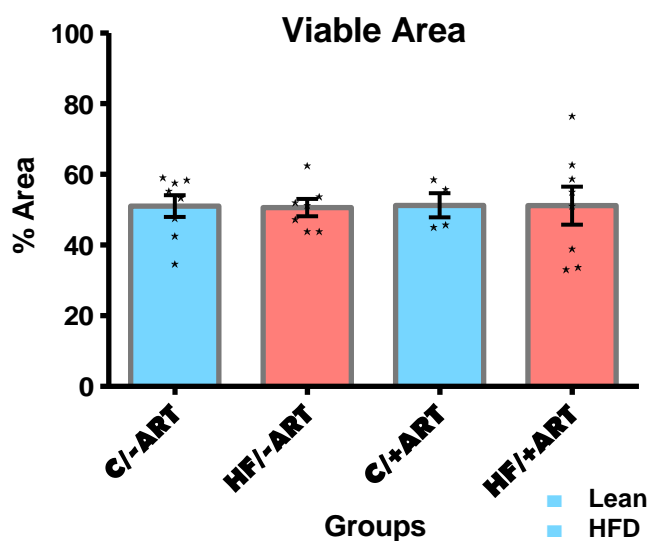
### 3.5.2. Regional Ischaemia-Reperfusion

The infarct size (expressed as a % of the AR) was determined after a regional ischaemic insult of 35 minutes. At the end of the stabilisation period of 30 minutes the heart was ligated for the 35-minute regional ischaemic insult and subsequently allowed to reperfuse for 1 hour by loosening the ligation (see chapter 2, section 2.2.15.3). After the full regional ischaemia-reperfusion protocol hearts were stained and infarct size determined as discussed in detail in chapter 2 (section 2.2.15.4).

See **Appendix F** for Haemodynamic Data for Regional Ischaemia-Reperfusion)

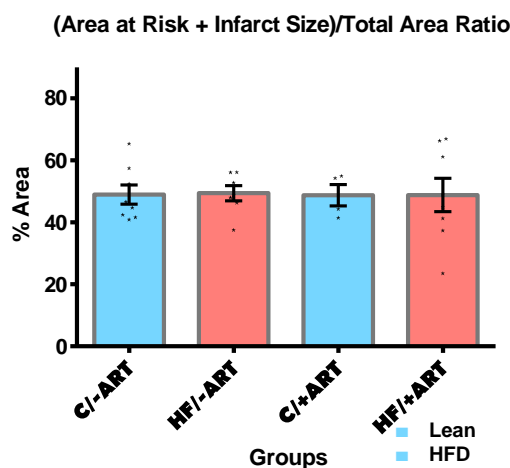
### 3.5.2.1. Infarct Size Determination

The mean VA was constant for all groups (between 40 % and 60 %) (**Fig. 3.25**).



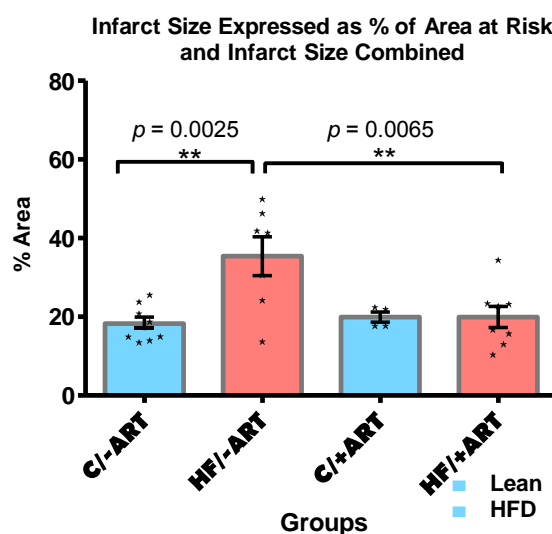
**Fig. 3.25.** Mean Viable Area / Group (expressed as % of total area) After Regional Ischaemia-Reperfusion (data expressed as mean  $\pm$  SEM / group). CI-ART: 51.03  $\pm$  3.10 %; HF/-ART: 50.60  $\pm$  2.46 %; CI/+ART: 51.25  $\pm$  3.44 %; HF/+ART: 51.18  $\pm$  5.39 %. N = 4-8 / group. N = 4 – 8 / group.

The mean AR was also similar between all groups (between 40 % and 60 %) (**Fig. 3.26**).



**Fig. 3.26.** Mean Area at Risk / Group (including Infarct Size) (expressed as % of total area) After Regional Ischaemia-Reperfusion (data expressed as mean  $\pm$  SEM / group). CI-ART: 48.97  $\pm$  3.10 %; HF/-ART: 49.40  $\pm$  2.47 %; CI/+ART: 48.75  $\pm$  3.44 %; HF/+ART: 48.83  $\pm$  5.39 %. N = 4 – 8 / group.

The mean infarct size was 18.3 % lower in the HF/-ART group compared to C/-ART ( $p = 0.0025$ ), and 20 % lower in the HF/+ART group compared to HF/-ART ( $p = 0.0065$ ). No further differences were observed (**Fig. 3.27**).



**Fig. 3.27.** Mean Infarct Size (expressed as a % of AR and Infarct Size Combined) After Regional Ischaemia-Reperfusion (data expressed as mean  $\pm$  SEM / group). C/-ART: 18.26  $\pm$  1.66 %; HF/-ART: HF/-ART: 35.40  $\pm$  4.95 %; C/+ART: 19.91  $\pm$  1.30 %; HF/+ART: 19.95  $\pm$  2.70 %. N = 4 – 8 / group.

### 3.6. Biochemical Analysis – Western Blot Analysis

For pre-ischaemia-reperfusion (baseline) measurements, hearts of fasted animals were excised, weighed and snap-frozen in liquid nitrogen for subsequent Western blot analyses, whilst for the post-ischaemia-reperfusion measurements, hearts were snap-frozen at the end of the global ischaemia/perfusion protocol for subsequent Western blot analyses.

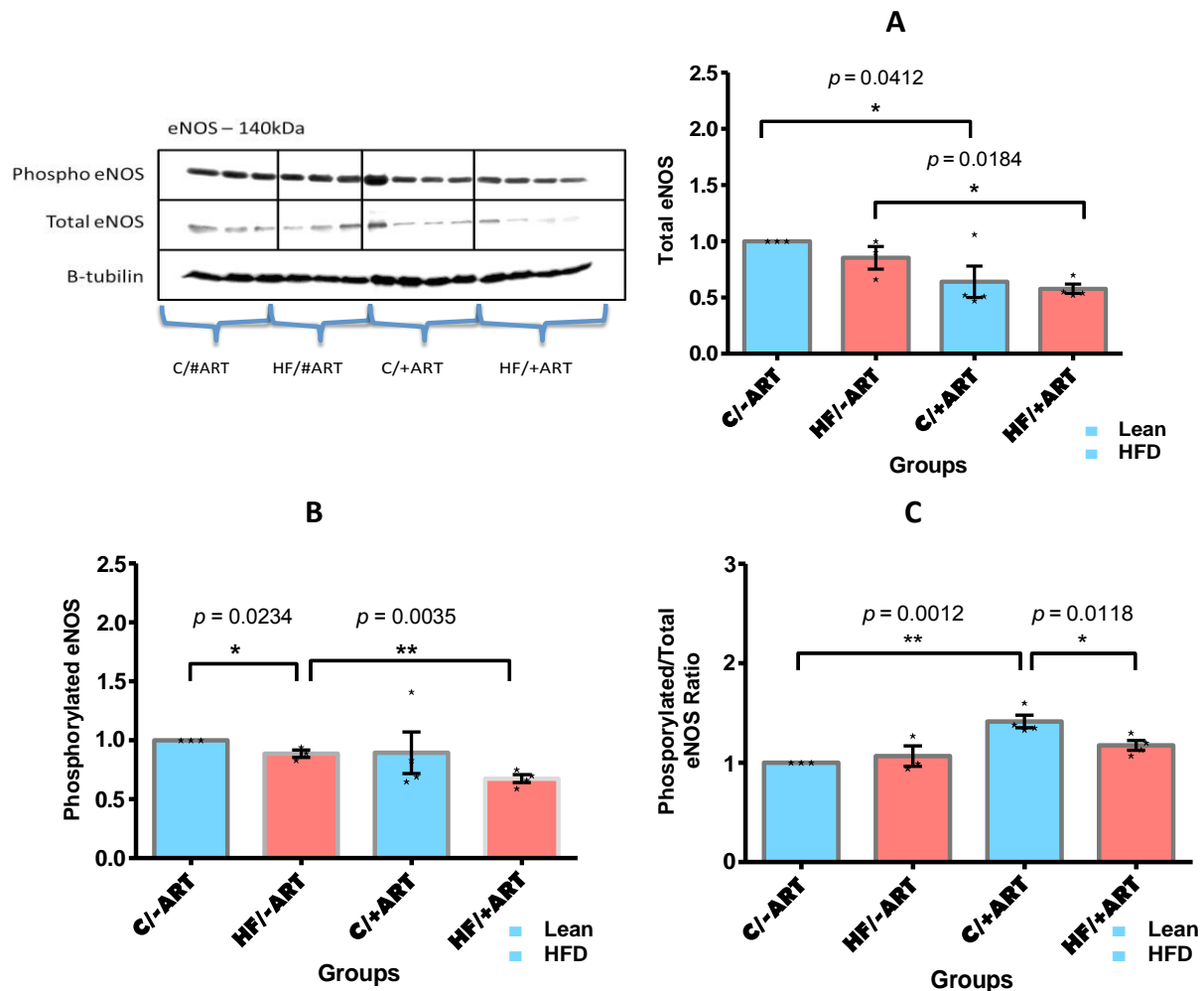
The proteins analysed for both pre-ischaemia-reperfusion and post-ischaemia-reperfusion included:

- NOS signalling: Total- and phosphorylated eNOS, total- and phosphorylated PKB/Akt and total- and phosphorylated AMPK
- ROS signalling: Total nitrotyrosine and total p22 Phox
- Inflammatory signalling: Total I $\kappa$ B $\alpha$

### 3.6.1. Nitric Oxide Synthase (NOS) Signalling

#### 3.6.1.1. Endothelial Nitric Oxide Synthase (eNOS)

##### 3.6.1.1.1. eNOS: Pre-ischaemia-Reperfusion

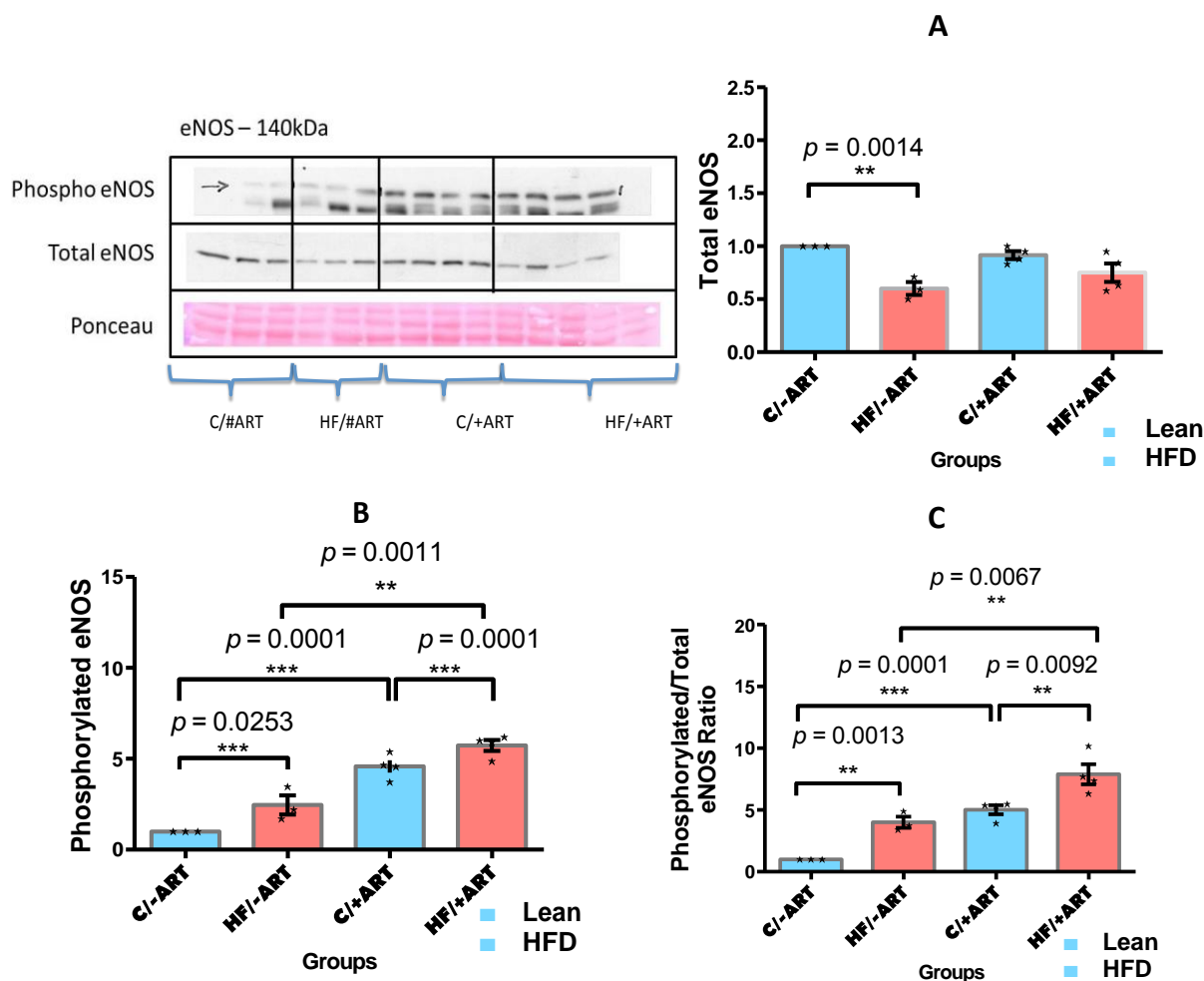


**Fig. 3.28 A- C.** eNOS: Pre-Ischaemia-Reperfusion - Expressed as a Ratio of C/-ART (= 1) (data expressed as mean ± SEM / group). **A.** Total eNOS: C/-ART: 1.00; HF/-ART: 0.85 ± 0.10; C/+ART: 0.64 ± 0.14 and HF/+ART: 0.58 ± 0.04. **B.** Phosphorylated eNOS: C/-ART: 1.00; HF/-ART: 0.89 ± 0.03; C/+ART: 0.90 ± 0.18 and HF/+ART: 0.68 ± 0.03. **C.** Phosphorylated/Total eNOS Ratio: C/-ART: 1.00; HF/-ART: 1.07 ± 0.10; C/+ART: 1.42 ± 0.06 and HF/+ART: 1.18 ± 0.05. N = 3 - 4 / group.

Total eNOS expression was 36 % lower in the C/+ART compared to C/-ART group ( $p = 0.0412$ ) and 58 % in the HF/+ART compared to HF/-ART group ( $p = 0.0184$ ) (**Fig. 3.28 A**). Phosphorylated eNOS decreased 11 % in the HF/-ART compared to C/-ART group ( $p =$

0.0234) and 21 % in the HF/+ART compared to HF/-ART group ( $p = 0.0035$ ) (**Fig. 3.28 B**). The phosphorylated/total ratio of eNOS was 42 % greater in the C/+ART compared to C/-ART group ( $p = 0.0012$ ), and 17 % lower in the HF/+ART compared to the C/+ART group ( $p = 0.0118$ ) (**Fig. 3.28 C**). No further differences were observed.

### 3.6.1.1.2. eNOS: Post-Ischaemia-Reperfusion

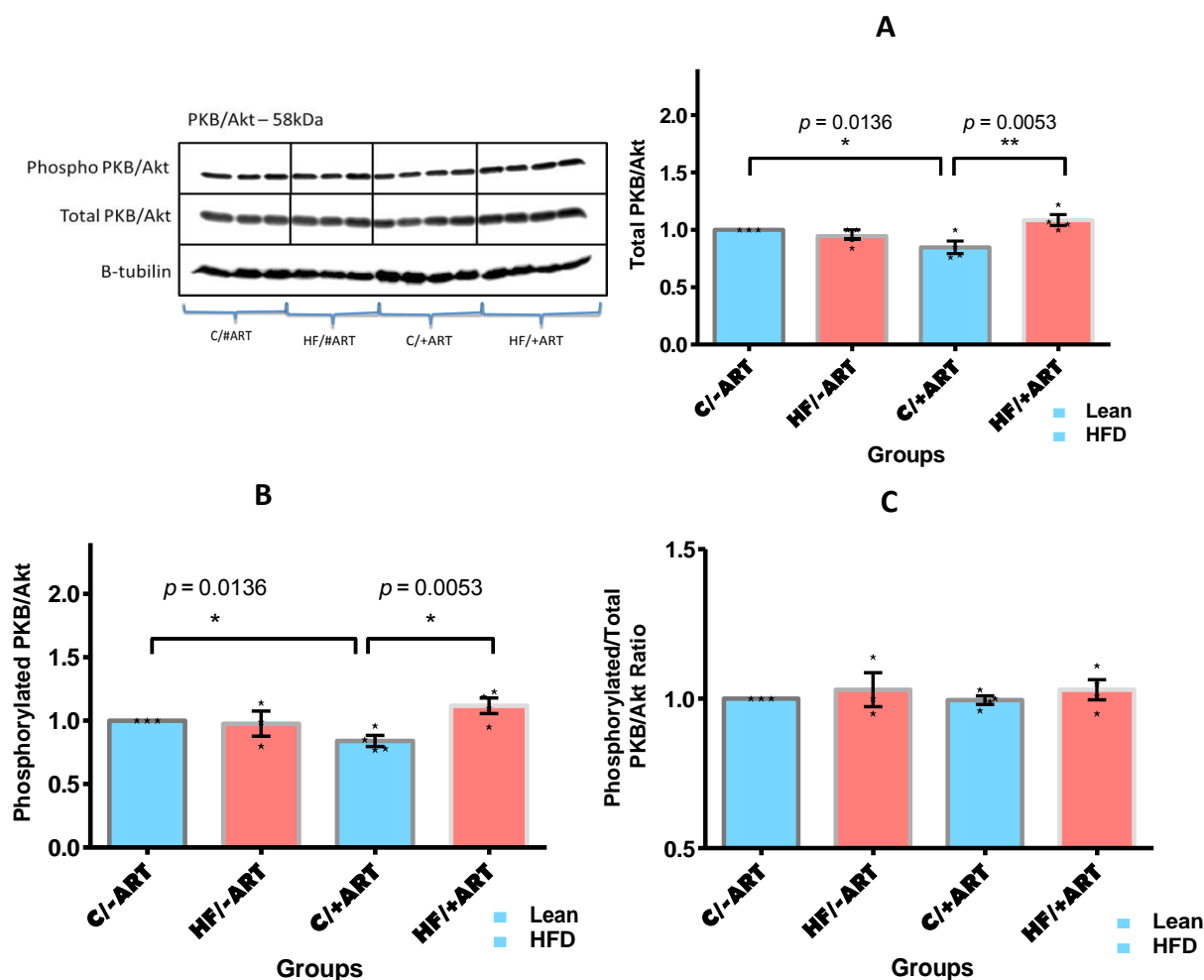


**Fig. 3.29 A - C.** eNOS: Post-Ischaemia-Reperfusion - Expressed as a Ratio of C/-ART (= 1) (data expressed as mean ± SEM / group). **A.** Total eNOS: C/-ART: 1.00; HF/-ART: 0.60 ± 0.061; C/+ART: 0.92 ± 0.038 and HF/+ART: 0.75 ± 0.088. **B.** Phosphorylated eNOS: C/-ART: 1.00; HF/-ART: 2.46 ± 0.53; C/+ART: 4.58 ± 0.34 and HF/+ART: 5.73 ± 0.30. **C.** Phosphorylated/Total eNOS Ratio: C/-ART: 1.00; HF/-ART: 4.00 ± 0.45; C/+ART: 5.02 ± 0.37 and HF/+ART: 7.89 ± 0.82. N = 3 - 4 / group.

Total eNOS expression was 40 % lower in the HF/-ART compared to C/-ART group ( $p = 0.0014$ ) (**Fig. 3.29 A**). Phosphorylated eNOS increased 2.46-fold in the HF/-ART ( $p = 0.0253$ ) and 3.58-fold in the C/+ART ( $p = 0.0001$ ) compared to the C/-ART group. Phosphorylated eNOS levels were 2.3-fold higher in the HF/+ART compared to HF/-ART group ( $p = 0.0011$ ) and 25 % higher compared to the C/+ART group ( $p = 0.0001$ ) (**Fig. 3.29 B**). Phosphorylated/total eNOS ratio increased 4-fold in the HF/-ART ( $p = 0.0013$ ) and 5.02-fold in the C/+ART ( $p = 0.0001$ ) compared to C/-ART group. The phosphorylated/total eNOS ratio was 2-fold higher in the HF/+ART compared to HF/-ART group ( $p = 0.0067$ ) and 57 % higher compared to the C/+ART group ( $p = 0.0092$ ) (**Fig. 3.29 C**). No further differences were observed.

### 3.6.1.2. Protein Kinase B (PKB/Akt)

#### 3.6.1.2.1. PKB/Akt: Pre-Ischaemia-Reperfusion

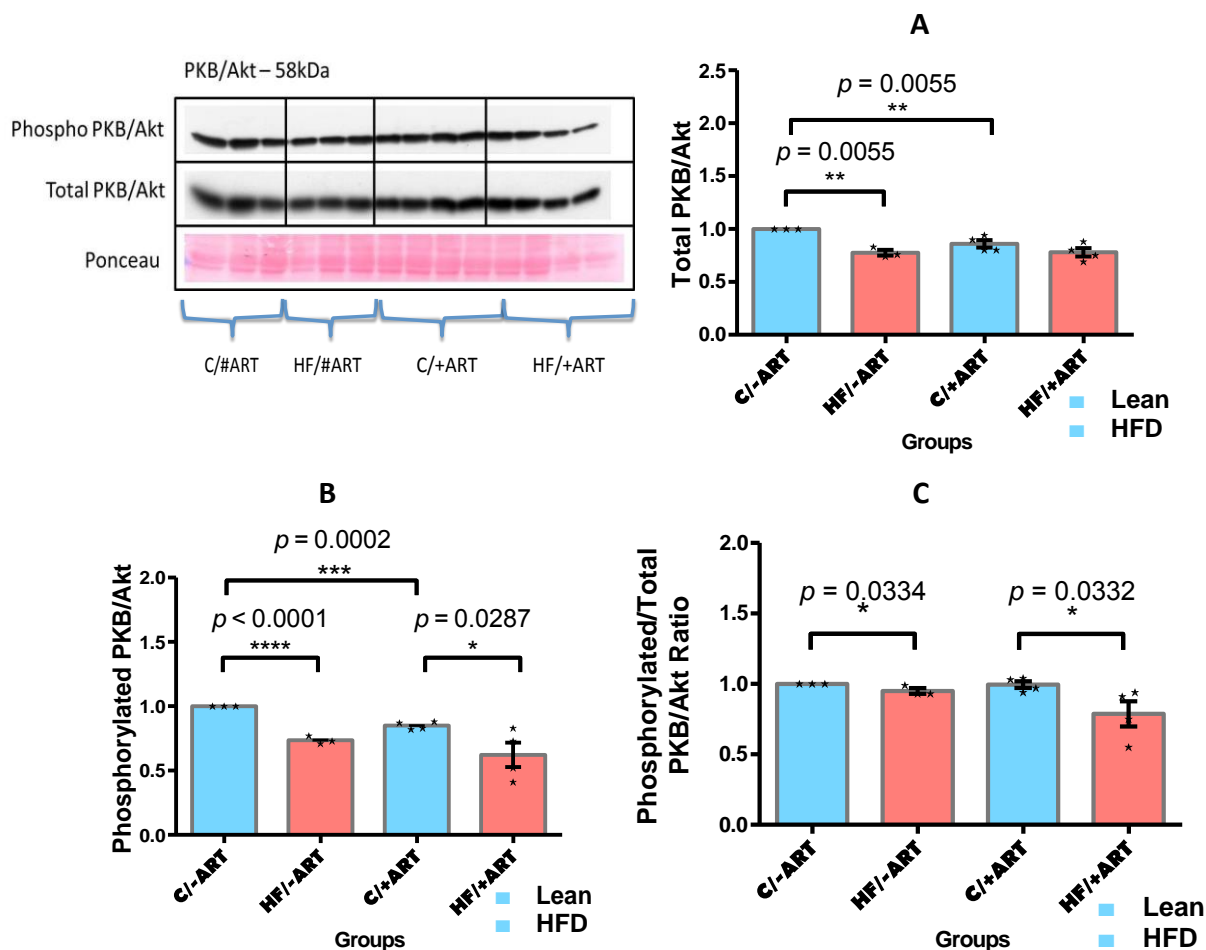


**Fig. 3.30 A - C. PKB/Akt: Pre-Ischaemia-Reperfusion** - Expressed as a Ratio of C/-ART (= 1) (data expressed as mean  $\pm$  SEM / group). **A.** Total PKB/Akt: C/-ART: 1.00; HF/-ART: 0.95  $\pm$  0.05; C/+ART: 0.85  $\pm$  0.05 and HF/+ART: 1.09  $\pm$  0.05. **B.** Phosphorylated PKB/Akt: C/-ART: 1.00; HF/-ART: 0.98  $\pm$  0.10; C/+ART: 0.84  $\pm$  0.04 and HF/+ART: 1.12  $\pm$  0.06. **C.** Phosphorylated/Total PKB/Akt Ratio: C/-ART: 1.00; HF/-ART: 1.03  $\pm$  0.06; C/+ART: 1.00  $\pm$  0.01 and HF/+ART: 1.03  $\pm$  0.03. N = 3 - 4 / group.

Total PKB/Akt decreased 15 % in the C/+ART compared to C/-ART group ( $p = 0.0136$ ). Total PKB/Akt increased 24 % in the HF/+ART compared to C/+ART group ( $p = 0.0053$ ) (**Fig. 3.30 A**). Phosphorylated eNOS increased 28 % in the HF/+ART compared to C/+ART group ( $p = 0.0238$ ), and decreased 16 % in the C/+ART group compared to C/-

ART (**Fig. 3.30 B**). No differences were observed in phosphorylated/total PKB/Akt ratios between any of the groups (**Fig. 3.30 C**).

### 3.6.1.2.2. PKB/Akt: Post-Ischaemia-Reperfusion



**Fig. 3.31 A - C. PKB/Akt: Post-Ischaemia-Reperfusion - Expressed as a Ratio of C/-ART (= 1) (data expressed as mean  $\pm$  SEM / group). **A.** Total PKB/Akt: C/-ART: 1.00; HF/-ART: 0.78  $\pm$  0.03; C/+ART: 0.86  $\pm$  0.04 and HF/+ART: 0.78  $\pm$  0.04. **B.** Phosphorylated PKB/Akt: C/-ART: 1.00; HF/-ART: 0.74  $\pm$  0.02; C/+ART: 0.85  $\pm$  0.01 and HF/+ART: 0.62  $\pm$  0.10. **C.** Phosphorylated/Total PKB/Akt Ratio: C/-ART: 1.00; HF/-ART: 0.95  $\pm$  0.02; C/+ART: 1.00  $\pm$  0.02 and HF/+ART: 0.78  $\pm$  0.09. N = 3 - 4 / group.**

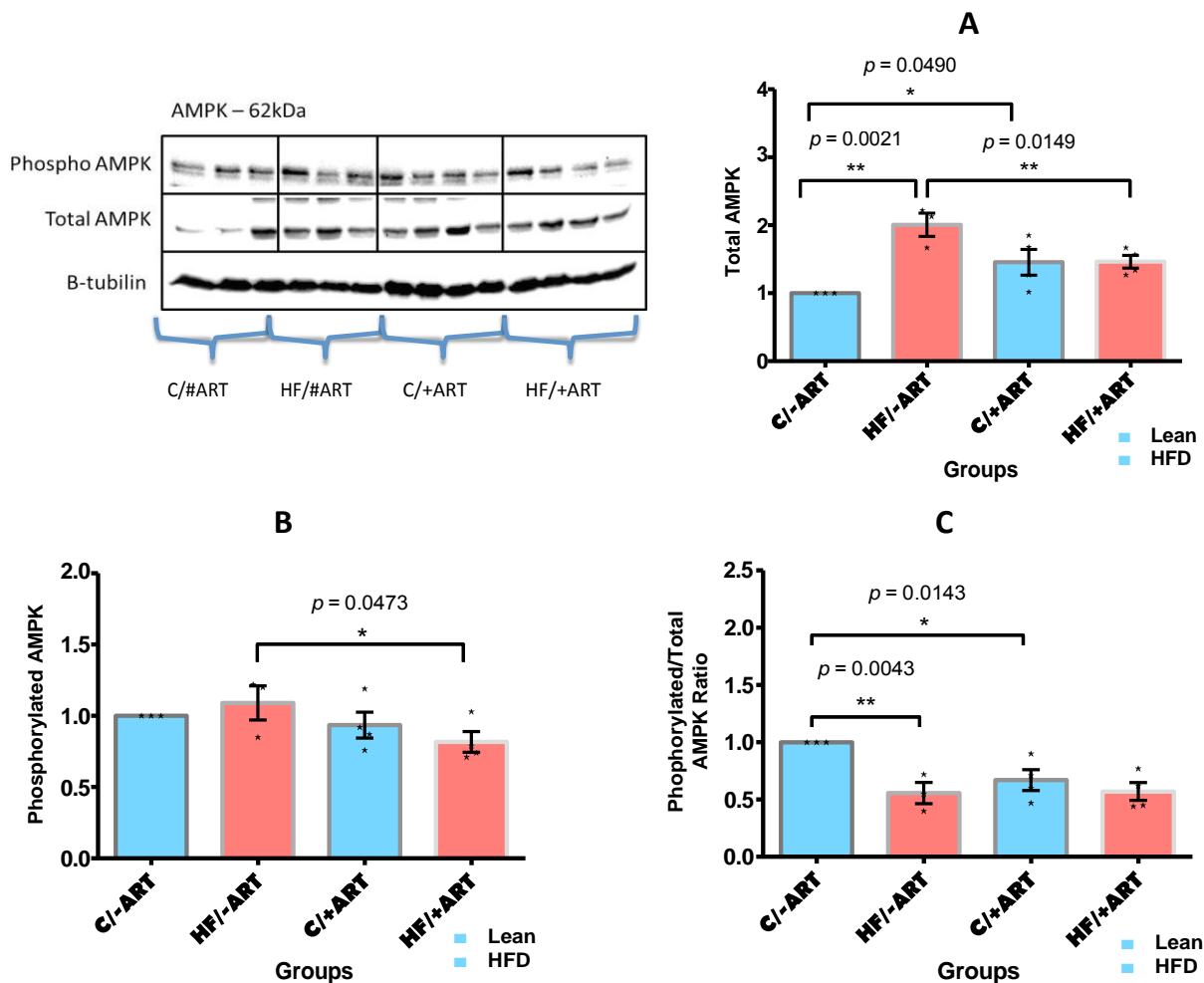
Total PKB/Akt decreased 22 % in the HF/-ART ( $p = 0.0055$ ) and 14 % in the C/+ART ( $p = 0.0055$ ) compared to C/-ART group (**Fig. 3.31 A**). Phosphorylated PKB/Akt decreased 26 % in the HF/-ART ( $p < 0.0001$ ) and 15 % in the C/+ART ( $p = 0.0002$ ) compared to C/-ART



group, and 23 % in the HF/+ART group compared to C/+ART (**Fig. 3.31 B**). Phosphorylated/total PKB/Akt decreased by 5 % in the HF/-ART compared to C/-ART group ( $p = 0.0334$ ) and 22 % in the HF/+ART compared to C/+ART group ( $p = 0.0332$ ) (**Fig. 3.31 C**). No further differences were observed.

### 3.6.1.3. Adenosine Monophosphate Kinase (AMPK)

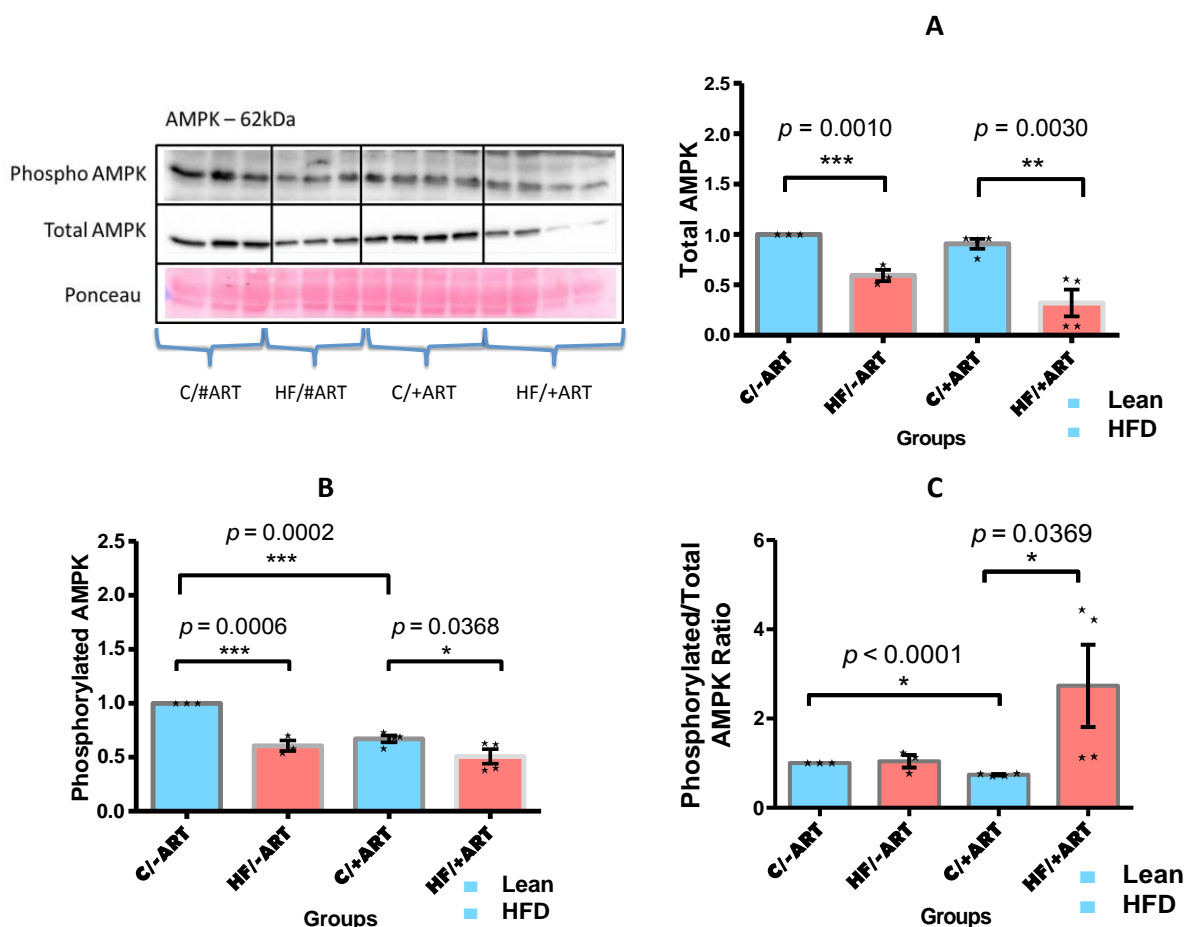
#### 3.6.1.3.1. AMPK: Pre-Ischaemia-Reperfusion



**Fig. 3.32 A - C.** AMPK: Pre-Ischaemia-Reperfusion - Expressed as a Ratio of C/-ART (= 1) (data expressed as mean  $\pm$  SEM / group). **A.** Total AMPK: C/-ART: 1.00; HF/-ART:  $2.01 \pm 0.17$ ; C/+ART:  $1.46 \pm 0.19$  and HF/+ART:  $1.46 \pm 0.09$ . **B.** Phosphorylated AMPK: C/-ART: 1.00; HF/-ART:  $1.09 \pm 0.12$ ; C/+ART:  $0.94 \pm 0.09$  and HF/+ART:  $0.82 \pm 0.07$ . **C.** Phosphorylated/Total AMPK ratio: C/-ART: 1.00; HF/-ART:  $0.56 \pm 0.09$ ; C/+ART:  $0.67 \pm 0.09$  and HF/+ART:  $0.57 \pm 0.08$ . N = 3 - 4 / group.

Total AMPK increased 2.01-fold in the HF/-ART ( $p = 0.0021$ ) and 46 % in the C/+ART ( $p = 0.049$ ) compared to the C/-ART group. Total AMPK decreased 55 % in the HF/+ART compared to HF/-ART group ( $p = 0.0149$ ) (**Fig. 3.32 A**). Phosphorylated AMPK decreased 27 % in the HF/+ART compared to HF/-ART group ( $p = 0.0473$ ) (**Fig. 3.32 B**). Phosphorylated/total AMPK ratio decreased 44 % in the HF/-ART ( $p = 0.0043$ ) and 33 % in the C/+ART ( $p = 0.0143$ ) compared to the C/-ART group (**Fig. 3.32 C**). No further differences were observed.

### 3.6.1.3.2. AMPK: Post-Ischaemia-Reperfusion



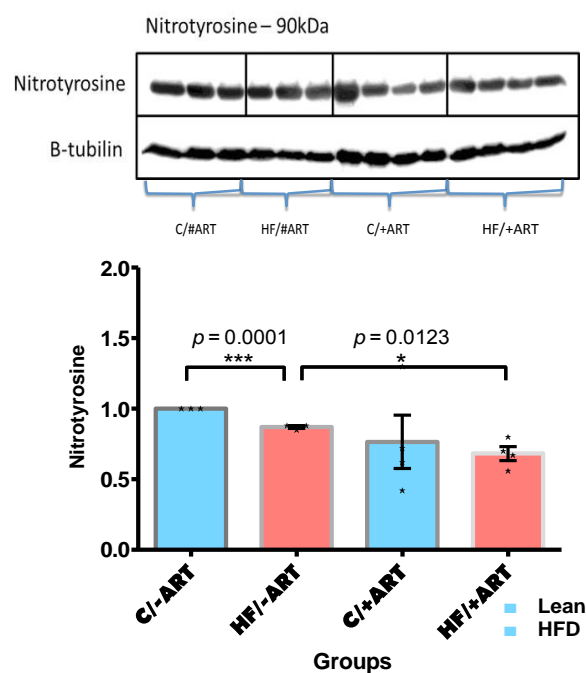
**Fig. 3.33 A - C.** AMPK: Post-Ischaemia-Reperfusion - Expressed as a Ratio of C/-ART (= 1) (data expressed as mean ± SEM / group). **A.** Total AMPK: C/-ART: 1.00; HF/-ART: 0.59 ± 0.06; C/+ART: 0.91 ± 0.05 and HF/+ART: 0.32 ± 0.13. **B.** Phosphorylated AMPK: C/-ART: 1.00; HF/-ART: 0.61 ± 0.05; C/+ART: 0.67 ± 0.03 and HF/+ART: 0.51 ± 0.07. **C.** Phosphorylated/Total AMPK ratio: C/-ART: 1.00; HF/-ART: 1.04 ± 0.14; C/+ART: 0.74 ± 0.01 and HF/+ART: 2.74 ± 0.92. N = 3 - 4 / group.

Total AMPK decreased 41 % in the HF/-ART compared to C/-ART group ( $p = 0.001$ ) and 59 % in the HF/+ART compared to C/+ART group ( $p = 0.003$ ). (**Fig. 3.33 A**). Phosphorylated AMPK decreased 39 % in the HF/-ART ( $p = 0.0006$ ) and 33 % in the C/+ART ( $p = 0.0002$ ) compared to C/-ART group. Phosphorylated AMPK also decreased 16 % in the HF/+ART compared to C/+ART group ( $p = 0.0368$ ) (**Fig. 3.33 B**). Phosphorylated/total AMPK ratio decreased 26 % in the C/+ART compared to C/-ART group ( $p = 0.0369$ ), but increased 3.70-fold in the HF/+ART compared to C/+ART group ( $p = 0.0369$ ) (**Fig. 3.33 C**). No further differences were observed.

### 3.6.2. Indicators of Oxidative Stress

#### 3.6.2.1. Nitrotyrosine

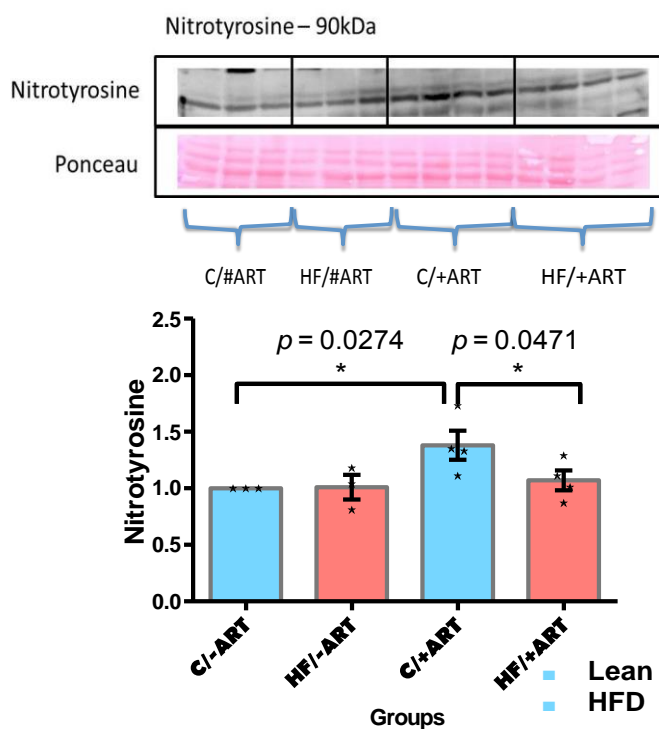
##### 3.6.2.1.1. Nitrotyrosine: Pre-Ischaemia-Reperfusion



**Fig. 3.34.** Nitrotyrosine: Pre-Ischaemia-Reperfusion - Expressed as a Ratio of C/-ART (= 1) (data expressed as mean  $\pm$  SEM / group). Nitrotyrosine: C/-ART: 1.00; HF/-ART: 0.87  $\pm$  0.01; C/+ART: 0.77  $\pm$  0.19 and HF/+ART: 0.69  $\pm$  0.05. N = 3 - 4 / group.

Total nitrotyrosine decreased 13 % in the HF/-ART compared to C/-ART group ( $p = 0.0001$ ) and 18 % in the HF/+ART compared to HF/-ART group ( $p = 0.0123$ ) (**Fig. 3.34**). No further differences were observed.

### 3.6.2.1.2. Nitrotyrosine: Post-Ischaemia-Reperfusion

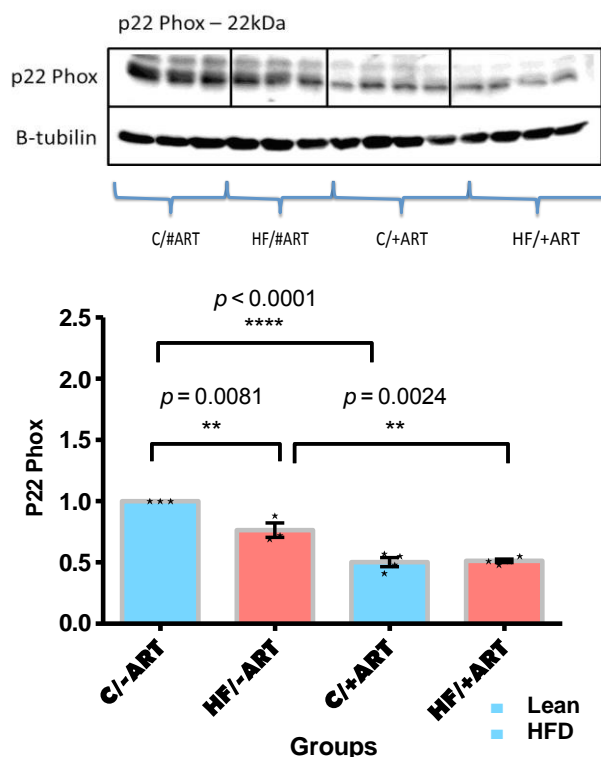


**Fig. 3.35.** Nitrotyrosine: Post-Ischaemia-Reperfusion - Expressed as a Ratio of C/-ART (= 1) (data expressed as mean  $\pm$  SEM / group). Nitrotyrosine: C/-ART: 1.00; HF/-ART: 1.01  $\pm$  0.11; C/+ART: 1.38  $\pm$  1.38 and HF/+ART: 1.07  $\pm$  0.09. N = 3 - 4 / group.

Total nitrotyrosine expression increased by 38 % in the C/+ART compared to C/-ART group ( $p = 0.0274$ ), but decreased 31 % in the HF/+ART compared to C/+ART group ( $p = 0.0471$ ) (**Fig. 3.35**). No further differences were observed.

### 3.6.2.2. P22 Phox Expression

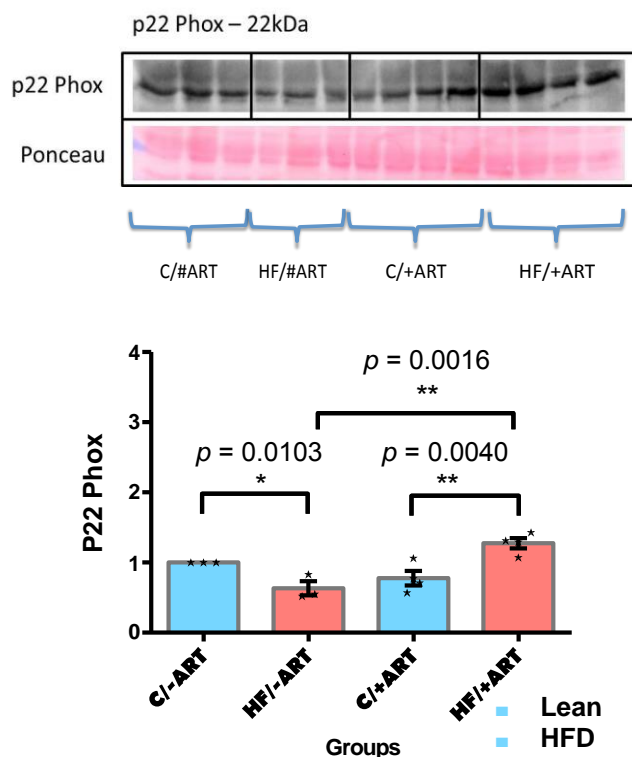
#### 3.6.2.2.1. P22 Phox Expression: Pre-Ischaemia-Reperfusion



**Fig. 3.36.** P22 Pho: Pre-Ischaemia-Reperfusion - Expressed as a Ratio of C/-ART (= 1) (data expressed as mean  $\pm$  SEM / group). P22 Phox: C/-ART: 1.00; HF/-ART:  $0.76 \pm 0.06$ ; C/+ART:  $0.50 \pm 0.04$  and HF/+ART:  $0.51 \pm 0.01$ . N = 3 - 4 / group.

Total p22 phox decreased 24 % in the HF/-ART ( $p = 0.0081$ ) and 50 % in the C/+ART ( $p < 0.0001$ ) compared to C/-ART group. Total p22 phox also decreased 25 % in the HF/+ART compared to HF/-ART group ( $p = 0.0024$ ) (**Fig. 3.36**). No further differences were observed.

### 3.6.2.2.2. P22 Phox Expression: Post Ischaemia-Reperfusion



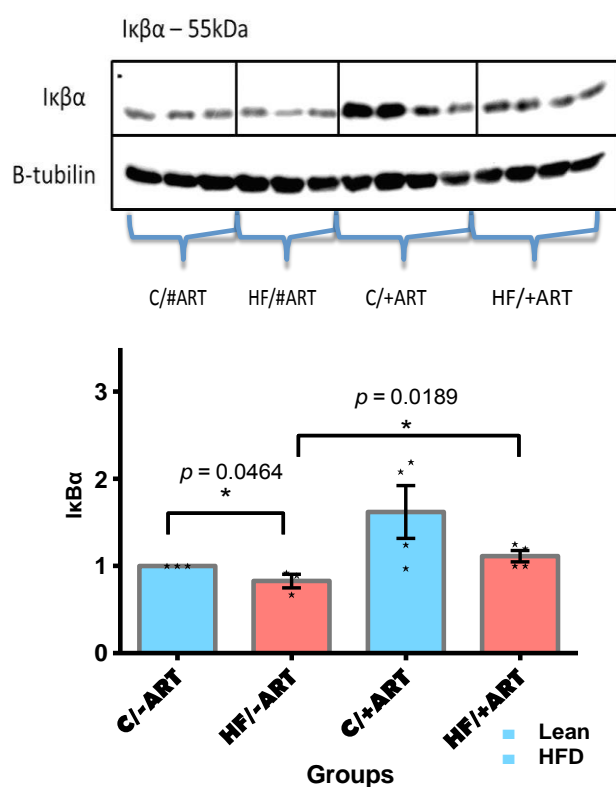
**Fig. 3.37.** P22 Phox: Post-Ischaemia-Reperfusion - Expressed as a Ratio of C/-ART (= 1) (data expressed as mean ± SEM / group). P22 Phox: C/-ART: 1.00; HF/-ART: 0.63 ± 0.10; C/+ART: 0.78 ± 0.10 and HF/+ART: 1.28 ± 0.08. N = 3 - 4 / group.

Total p22 phox decreased 37 % in the HF/-ART compared to C/-ART group ( $p = 0.0103$ ). Total p22 phox increased 65 % in the HF/+ART compared to HF/-ART group ( $p = 0.0016$ ) and 50 % in the HF/+ART compared to C/+ART group ( $p < 0.0040$ ) (**Fig. 3.37**). No further differences were observed.

### 3.6.3. Inflammatory Signalling

#### 3.6.3.1. Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B-cells Inhibitor, Alpha (I $\kappa$ B $\alpha$ )

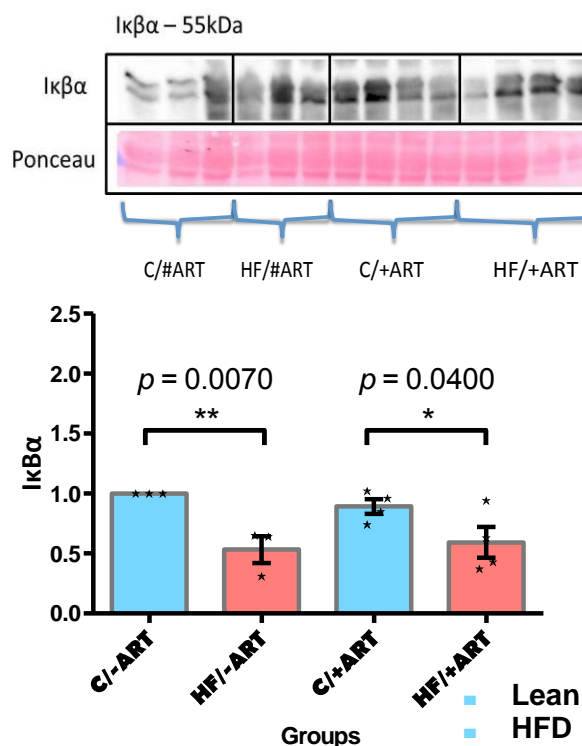
##### 3.6.3.1.1. I $\kappa$ B $\alpha$ : Pre-Ischaemia-Reperfusion



**Fig. 3.38.** I $\kappa$ B $\alpha$ : Pre-Ischaemia-Reperfusion - Expressed as a Ratio of C/-ART (= 1) (data expressed as mean  $\pm$  SEM / group). I $\kappa$ B $\alpha$ : C/-ART: 1.00; HF/-ART: 0.83  $\pm$  0.08; C/+ART: 1.62  $\pm$  0.30 and HF/+ART: 1.11  $\pm$  0.07. N = 3 - 4 / group.

Total I $\kappa$ B $\alpha$  decreased 14 % in the HF/-ART compared to C/-ART group ( $p = 0.0464$ ), and increased 28 % in the HF/+ART compared to HF/-ART group ( $p = 0.0189$ ) (**Fig. 3.38**). No further differences were observed.

### 3.6.3.1.2. I $\kappa$ B $\alpha$ : Post-Ischaemia-Reperfusion



**Fig. 3.39.** I $\kappa$ B $\alpha$ : Post-Ischaemia-Reperfusion - Expressed as a Ratio of C/ART (= 1) (data expressed as mean  $\pm$  SEM / group). I $\kappa$ B $\alpha$ : C/ART: 1.00; HF/ART: 0.53  $\pm$  0.11; C/+ART: 0.89  $\pm$  0.06 and HF/+ART: 0.59  $\pm$  0.13. N = 3 - 4 / group.

Total I $\kappa$ B $\alpha$  decreased 47 % in the HF/ART compared to C/ART group ( $p = 0.007$ ) and 30 % in the HF/+ART compared to C/+ART group ( $p = 0.04$ ) (**Fig. 3.40**). No further differences were observed.



## Chapter 4 – Discussion

### 4.1. Feeding Programme and Biometric Measurements

**Table 4.1.** Summary of the Effects of Different Experimental Conditions on Various Parameters in the Research Animals. **Symbols:** ↑↑ = Increase; ↓↓ = Decrease; - = No Effect.

Parameter		C/-ART	HF/-ART	C/+ART	HF/+ART
During Feeding- and Drug Treatment Programme	TBM (g)	-	↓(vs. C/-ART, at week 12)	-	↓(vs. C/+ART, at week 7)
	Rat Chow Consumption	-	"(vs. C/-ART)	-	"(vs. C/+ART)
	Water Consumption	-	"(vs. C/-ART)	-	"(vs. C/+ART)
On Day of Sacrifice	TBM (g)	-	↓(vs. C/-ART)	-	↓(vs. C/+ART)
	IP Fat Mass (%)	-	↓(vs. C/-ART)	-	↓(vs. C/+ART)
	Heart Mass (%)	-	-	-	-
	Heart Mass (g)	-	↓(vs. C/-ART)	-	-
	Liver Mass (%)	-	-	-	↓(vs. HF/-ART)

#### 4.1.1. Food and Water Consumption

Normal rat chow and water consumption remained constant within each experimental group during the first 31 days of the drug treatment programme. ART had no effect on the food and water consumption when comparing the treated groups (C/+ART and HF/+ART) with their respective untreated counterparts (C/-ART and HF/-ART) (*see Fig. 3.2 – 3.3*). Animals on the HFD received normal rat chow plus the additional HFD food mixture, and their normal rat chow consumption was significantly reduced compared to their respective lean, controls (*see Fig. 3.2*). It therefore appears that the animals in these groups preferred the sweet HFD food compared to the normal rat chow. As the HFD food was freely available to the HFD groups, rats mostly consumed the sweet HFD food while the lean, control animals only had access to normal rat chow. This observation is consistent with literature that shows that rodents have a preference for sweet foods rather than normal rat chow (Avena *et al.*, 2008; Lenoir *et al.*, 2007).

Interestingly, as seen in **Table 4.1**, the water consumption for HFD groups was lower compared to their respective lean controls (with and without ART) (**see Fig. 3.3**). This finding is in contrast to another study, where a diet (containing 15 % kcal sugar, 35 % kcal lard and 50 % kcal standard rat chow) was fed to rats and water consumption monitored over a period of 24 days (La Fleur *et al.*, 2014). They found that the rats on the high fat diets, consumed more water than the rats that were fed normal rat chow. The decrease in water consumption between the HFD groups compared to the lean, control groups in the present study can possibly be attributed to the higher content of water in the HFD food. Studies have also inversely associated water consumption with energy intake and positively associated increased water consumption with high fibre intake (Kant AK *et al.*, 2009), which supports our findings.

As seen in **Table 4.1**, ART or its combination with a HFD had no effect on food and water consumption. These findings are regarded novel as this is the first study that uses this specific ART combination in a rat model of obesity. According to literature, ART *per se* mostly affects food and water intake through ART-associated side effects including nausea, vomiting and other gastrointestinal complications. Adverse effects also differ between types of drugs, drug classes, dosage and combinations of ART drugs (Shevitz and Knox, 2001).

#### **4.1.2. Biometric Measurements During and After the Feeding and Drug Treatment Programme**

##### **4.1.2.1. Total Body Mass (TBM) and Intra-Peritoneal (IP) Fat Mass**

The mean TBM of the four experimental groups were identical at the start of the feeding programme (**Fig. 3.5**). The mean TBM of the HFD groups (with and without ART) showed a greater increase than the lean, control groups (with and without ART) indicating that the HFD was successful in causing significant body mass increase compared to the lean, control groups (**Fig. 3.6 and 3.7**). The increase in mean TBM continued for the duration of the feeding programme, including the final six weeks after addition of the ART drug treatment programme and was maintained until the day of sacrifice (**Fig. 3.8**).

The increase in mean TMB corresponds with previous data from our laboratory that used a similar diet and feeding period (Salie *et al.*, 2014; Huisamen and Flepisi, 2014). Also, another study by Zou *et al.*, 2006 found similar final mean TBM's in lean (375.63 g; weight gain 139.0 g) and HFD groups (433.6 g; weight gain 189.3 g) compared to this study, although their feeding programme consisted of the administration of a high fat emulsion (10 mg / kg; 4342 kcal / L) via oral gavage and free access to saccharose (18 %) solution for 6 weeks.

As seen in **Table 4.1**, ART drug treatment had no effects on mean TBM increases in either, the lean control animals or the animals on the HFD (**Fig. 3.5 - 3.7**). Only a few studies have described the effects of ART on body mass *per se* and findings are often contradictory with HIV-associated weight loss, still prevalent in HIV-populations (Wanke *et al.*, 2000). Nonetheless, it has been shown that ART can reverse HIV/AIDS-associated weight loss, and obesity and lipodystrophy (peripheral lipoatrophy and abdominal lipohypertrophy in particular) are becoming more prevalent in HIV-infected populations using ART (Mulligan *et al.*, 2005; Malewa *et al.*, 2008). Also, metabolic dysregulation in PLWH on ART is on the rise and usually associated with prolonged ART-use (Islam *et al.*, 2012; Anderson *et al.*, 2004; Fedele *et al.*, 2011). The exact mechanistic interplay between a HFD, ART and obesity is not well known (Islam *et al.*, 2012; Fedele *et al.*, 2011; Mulligan *et al.*, 2001), but it appears that a combination of factors may contribute to increased TBM in PLWH. The hepatotoxic and toxic effect of ART-treatment on adipose tissue (in combination with metabolic abnormalities associated with a HFD and obesity) may contribute to an increased adiposity (especially in the abdominal area) and/or decreased adiposity (in the limb area) in PLWH (de Waal *et al.*, 2013). Also, ART can contribute to hepatomegaly through the possible overlap of pathophysiological pathways and indirectly contribute to an increased TBM through metabolic dysregulation (e.g. through ART-associated lipodystrophy) (de Waal *et al.*, 2013).

The data of the present study demonstrated that the fixed ART drug combination had no effect on the TBM gains observed in both the lean, control and HFD groups. However, it is possible that longer ART exposure (beyond the treatment period of 6 weeks) may have more profound effects on the metabolic regulation of adiposity and subsequently could result in additional increases in mean TBM. Further studies investigating this possibility may shed more light. NRTIs have also been shown to be less toxic to adipose tissue than PIs (Estrada *et al.*, 2006; Silva *et al.*, 1998). The specific drug combination used in the

present study may also explain why the TBM of the treated groups was not affected, as EFV (contained in Odimmune™) has previously been associated with limb fat loss (lipohypotrophy) due to its toxic effect on adipocytes (de Waal *et al.*, 2013) rather than lipohypertrophy (Estrada *et al.*, 2002). Studies have also shown better health outcomes in terms of medication adherence and reduced adverse effects when PIs are replaced by NRTIs in combination ART (Carr *et al.*, 2003). This indicates that NRTI-associated adverse effects may affect health outcomes in general to a lesser extent than PIs.

The ability of the HFD programme to induce a rat model of obesity was further validated by the significant increases observed in the mean IP fat mass % in the HFD groups compared to their respective lean, controls (**Fig. 3.9**). The mean IP fat mass was 60.22 % higher in the HF/-ART vs. C/-ART groups, and 91.26 % higher in the HFD/+ART vs. C/+ART groups respectively. This increase in mean IP fat mass associated with the HFD corresponded with those observed in other studies (Salie *et al.*, 2014; Huisamen and Flepisi, 2014; Cole *et al.*, 2011; Pancani *et al.*, 2013). In the present study, ART drug treatment did not exert any additional effects on IP fat mass in the HFD group. As far as we are aware, the effects of this specific fixed ART drug combination on IP fat mass in rats (both lean and diet-induced obese) has not previously been described and this data can therefore be considered novel.

Although the ART drug combination in our study did not exert any additional effects on IP fat mass, it has been reported that lipohypertrophy (especially in the abdominal area) is a major health concern in PLWH (Han S *et al.*, 2009; Van der Valk *et al.*, 2001). Mitochondrial toxicity of ARTs has been pointed out as a key factor that can contribute to limb lipoatrophy (De Waal *et al.*, 2013; Carr S, 2003; Freitas *et al.*, 2013). NRTIs have been mostly associated with limb-fat wasting though possible inhibition of mitochondrial DNA synthesis while NNRTIs are associated with both adipogenesis in the abdominal area (Flint *et al.*, 2009; Saint-Marc *et al.*, 2000; Freitas *et al.*, 2013) and promoting lipolysis in the limb area (Carr S, 2003; Freitas *et al.*, 2013). Many questions remain unanswered regarding the exact mechanisms and need further investigation.

To summarise: The HFD in our study successfully increased the mean TBM and mean IP fat mass of HFD groups compared to lean, control groups. The specific FDC of ART had no additional effects on either the mean TBM or IP fat mass, and this might be due to the either the combination, dosage or period of administration of the drugs. It seemed that the

IP fat mass increase was more pronounced when ART was administered in combination with our HFD (60 % increase in HF/-ART compared to C/-ART, and 91 % increase in HF/+ART compared to C/+ART), although this difference was not significant.

#### 4.1.2.2. Heart and Liver Mass

Although there was no difference in the mean heart mass (% of TBM) in any of the groups (**Fig. 3.10 B**) the absolute heart mass (expressed in g) of the HFD/-ART was 0.16 g higher compared to the C/-ART group ( $p = 0.036$ ) (**Fig. 3.10 B**). This represents a 10.30 % increase, while there was no difference in the heart mass (g) between the groups receiving ART. These findings are in agreement with those of other studies where the actual mean heart mass of rats receiving a high fat diet (although not similar in composition and feeding period) also increased, but when expressed as % of TBM no differences were observed (Cole *et al.*, 2011; Pancani *et al.*, 2013). Ouwens *et al.* (2005) did indeed find an increased heart / body mass ratio after the HFD feeding programme (25 % fat; 32 % protein, 25 % carbohydrate; 7 weeks), with a two-fold increase in cardiac TG content compared to the control group. Although TG content in cardiac tissue was not measured in our study, increased circulating TG levels were observed in the HFD groups compared to their respective lean, control groups (**Fig. 3.16**) and this is positively associated with cardiac hypertrophy in other studies (Ouwens *et al.*, 2005). ART-treatment *per se* had no effect on the mean heart mass in our study. Also, the HFD in combination with ART-treatment had no effect on the mean heart mass (**Fig. 3.10 A**). To our knowledge, this finding is novel in the context of our study, although ART drug combinations have been associated with lipodystrophy and dyslipidaemia that could lead to anthropometric abnormalities such as myocardial hypertrophy (Leite *et al.*, 2008; Keithley *et al.*, 2009; Grim *et al.*, 2010).

As seen in **Table 4.1**, the diet and the treatment *per se* had no effect on mean liver mass, but the combination of the HFD and ART increased the mean liver mass significantly (10.69 %) compared to the untreated HFD group (**Fig. 3.11**). A study by Zou *et al.* (2006) found an increase in mean liver mass (% of TBM) in the HFD group (3.46 %;  $N = 12$ ) compared to the lean, control (2.59 %;  $N = 12$ ) (feeding programme: gavaging animals with a high fat emulsion: 10 mg / kg; 4342 kcal / L and free access to saccharose (18 %) solution; 6 weeks). They also found increased hepatic lipid content compared to the lean control group. The reason for the elevated mean liver mass in the HF/+ART group

compared to the HF/-ART group can possibly be attributed to the combination of the hepatotoxic effect of ART (Sutinen *et al.*, 2002), combined with the effects of a HFD on liver function. Liver damage and non-alcoholic fatty liver disease are frequently seen in HIV-positive subjects on ART (Maida *et al.*, 2006; Maide *et al.*, 2006). NNTRTIs have been associated with liver injury, hepatotoxicity and hepatomegaly (Carr and Cooper *et al.*, 2000; Carr A *et al.*, 2000; Dieterich *et al.*, 2004). Increased liver mass appears to be a common feature in HIV-1 infected patients on ART (Moreno-Tores *et al.*, 2007), especially in PLWH on ART with lipodystrophy (Sutinen *et al.*, 2002). The reason for the increased liver mass in the HF/+ART group (compared to HF/-ART) in our study can thus possibly be explained by a combination of factors. These factors include a possible synergistic relationship between ART-associated hepatotoxicity and the HFD, possible intra-hepatic TG deposition in the liver (increased circulating TG levels in both the HF/-ART and HF/+ART were observed in the current study compared to lean, controls), and/or a combination of these factors.

To summarise: Obesity has been associated with the ectopic lipid deposition in the heart and liver (associated with increased liver and heart mass) (Tchernof and Després, 2013). In the present study, an increase in actual heart mass (g) was observed in the HFD group compared to the lean, control animals, with no changes in the liver mass. The increased heart mass observed in our study may be the result of enhanced ectopic lipid deposition, as indicated by increased circulating TG levels (see later). Increased cardiac work-load associated with obesity (Tchernof and Després, 2013) may have resulted in increased heart mass in our study and is further underscored by the increased expression of AMPK observed in the HF/-ART group compared to the C/-ART group in our study (Dyck, 2006; Miller *et al.*, 2005; Heinrich *et al.*, 2010). Increased expression of AMPK prepares the heart for a substantial increase in energy demand required during physical activity in obesity compared to lean (Dyck, 2006). During physical activity the increased expression of AMPK can lead to rapid activation when ATP levels drop, resulting in rapid compensation for energy demand. In our study, animals remained mostly sedentary and this could explain the increased AMPK expression but not activation as seen in our study.

On the other hand the ART administration in the HFD animals increased the mean liver mass (% of TBM) by 10.92 %. The effects of this ART drug combination in the context of obesity may have contributed to possible liver damage. These findings associated with ART-use in our study are also regarded as novel since the use of this ART combination in a HFD rat model of obesity has not yet been described.

## 4.2. Results of Biochemical Analyses – Glucose, Insulin and Lipid Levels.

**Table 4.2.** Summary of the Effects of Different Experimental Conditions on Various Biochemical Parameters in the Research Animals. Symbols: ↑↑ = Increase; ↓↓ = Decrease; - = No Effect.

Parameters (fasted)	C/-ART	HF/-ART	C/+ART	HF/+ART
Glucose Level (mmol/L)	-	-	-	-
Insulin (ng / ml)	-	-	↑(vs. HF/+ART and C/-ART)	-
TC (mmol/L)	-	-	↑(vs. HF/+ART)	-
HDL (mmol/L)	-	-	-	-
TG (mmol/L)	-	↑(vs. C/-ART)	-	↑(vs. C/+ART)
CD (mmol/L)	-	-	-	↑(vs. HF/-ART)
TBARS (μmol/L)	-	↑(vs. C/-ART)	↑(vs. C/-ART)	-

### 4.2.1. Glucose and Insulin Levels

As can be seen from the summary in **Table 4.2**, none of the experimental conditions exerted any effects on mean fasting glucose levels (**Fig. 3.12**). This is contradictory to other studies that found a difference in mean glucose levels after a similar 16-week HFD feeding programme (Salie *et al.*, 2014). A study by Zou *et al.* (2006) also found an increase in mean glucose levels (mmol / L) in animals receiving a HFD (8.36 mmol / L; N = 12) compared to the lean (5.36 mmol / L; N = 12) group in their study. On the other hand, Ouwens *et al.* (2005) found similar mean glucose levels to the current study in rats receiving a high fat, high carbohydrate diet for 7 weeks compared to lean, control. Also, Nduhirabandi *et al.* (2011) found no difference in mean glucose levels compared to lean, control after completing a similar HFD feeding programme (N = 6).

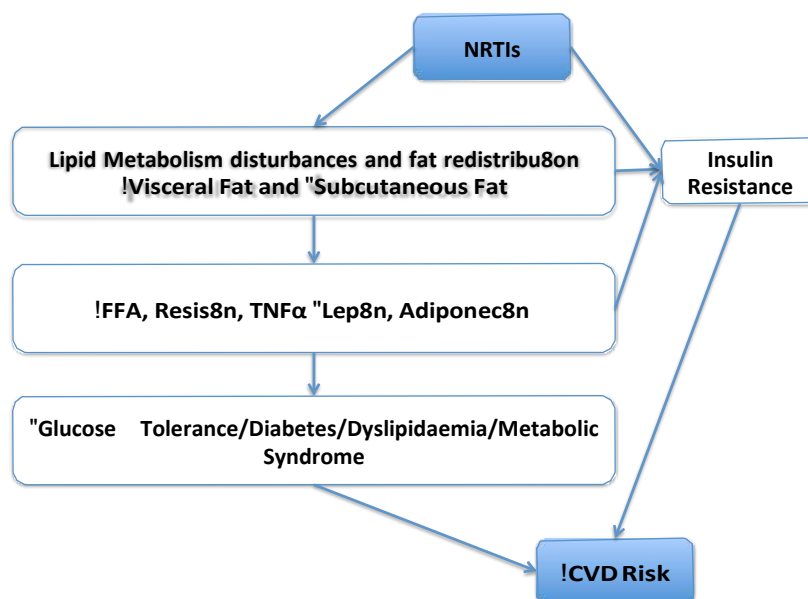
The HFD *per se* had also no effect on mean insulin levels in our study (**Fig. 3.13**) and was therefore unsuccessful in inducing insulin resistance. This finding was unexpected, as it is known that the risk for developing insulin resistance and diabetes increases with



increasing body weight. In support of our findings, a study by Ouwens *et al.* (2005) could also not show increased insulin levels after a HFD feeding programme. In contrast, Nduhirabandi *et al.* (2011) found a 245 % increase in serum insulin in the HFD group compared to lean, control (following a similar diet and period of feeding). In a study by Zou *et al.* (2006), an increase in insulin levels were also found in the HFD group compared to the lean group. In the study by Salie *et al.* (2014) insulin resistance was induced, but their increase in mean TBM during their feeding programme was approximately double than that of our study. The type of fat used in the HFD feeding programme has been shown to induce insulin resistance with variable results and may have also played a role (Beuttner *et al.*, 2006). In this respect, Beuttner *et al.* (2006) showed that the classic physiological effects of HF diets, including insulin resistance, manifested more prominently when lard and olive oil (mainly long-chain, saturated and monounsaturated fatty acids) were used in HF diets compared to HF diets containing coconut oil or Fish oils. It may be possible that if a greater TBM in the HFD groups were achieved either though following a different diet or a longer period of feeding beyond our 16-week feeding programme, a significant difference in fasting glucose levels between lean and HFD groups may have been observed.

In the present study, ART led to a marked reduction in insulin levels in the lean, control animals (see **Table 4.2**), where mean insulin levels were 73.02 % lower in the C/+ART group compared the C/-ART group and 75.61 % lower compared to the HF/+ART group (**Fig. 3.13**). This finding is difficult to explain, especially in view of the fact that the fasting glucose levels were unaffected in this group. However, previous studies have associated the use of ARTs with insulin resistance and insulin level dysregulation (Mayosi *et al.*, 2009; Signorini *et al.*, 2012; Islam *et al.*, 2012) (**Fig. 4.1**).





**Fig. 4.1.** *NRTIs Involvement in Metabolic Dysregulation Associated with Lipdystrophy and CVD.* **Abbreviations:** NRTIs: Nucleoside Reverse Transcriptase Inhibitors; CVD: Cardiovascular Disease; FFA: Free Fatty Acid; TNF $\alpha$ : Tumor Necrosis Factor Alpha. **Symbols:**  $\uparrow\uparrow$  = Increase;  $\downarrow\downarrow$  = Decrease; - = No Effect. (Freitas *et al.*, 2013).

The mechanism by which insulin level dysregulation associated with ART use is induced is not fully understood, but may involve inhibition of peripheral glucose uptake (inhibition of glucose transporter GLUT-4) and also inhibition of hepatic glucose production (Flint *et al.*, 2009). It has also been shown that the ability of insulin to suppress glucose production and lipolysis is reduced in PLWH on ART (Van der Valk *et al.*, 2001). Insulin resistance in patients on ART may furthermore be a result of chronic hypertriglyceridemia (Flint *et al.*, 2009; Malangu N, 2014). An increased mean TG level in the C/+ART group compared to HF/+ART was observed (see later) and thus, may have contributed to the reduced insulin level in the C/+ART group compared to HF/+ART. Pancreatitis is a known adverse effect of ART and NRTI and could affect insulin secretion (Moore *et al.*, 2001; Smith *et al.*, 2008). ART has also been associated with pancreatic  $\beta$ -cell dysfunction, mediated through numerous possible pathways that include pancreatic lipid deposition (associated with  $\beta$ -cell dysfunction and reduced pancreatic insulin secretion) (Domingo *et al.*, 2012; Haugard *et al.*, 2005; Pi *et al.*, 2010). Studies have further shown a decrease in insulin secretion associated with ART use and in particular with NRTIs (Maassen and Heine, 2007).

Although it is indeed possible that the ART exerted adverse or toxic effects on the pancreas leading to  $\beta$ -cell dysfunction and a subsequent reduction in serum insulin levels, the question remains why this was only observed in the C/+ART group. Future studies with larger sample sizes, and closer inspection of pancreas morphological changes may prove or disprove the current unexpected findings.

To summarise: although the HFD in the present study led to increased TBM and IP fat mass (obesity), it was unsuccessful in inducing insulin resistance. Overall, treatment with the ART drug failed to exert any additional effects; however, the surprisingly low insulin levels observed in the C/-ART group (in the absence of any changes in the other groups) warrants further investigations.

#### **4.2.2. Lipid Profile**

It is well known that obesity affects the lipid profile and can be associated with increased LDL, TC, and TG levels and decreased HDL levels (Alwan, 2011; Fedele *et al.*, 2011; Tchernof and Després, 2013). Furthermore, ART has previously been shown to affect the lipid profile and is associated with elevated TC, LDL, TG levels and reduced HDL levels (Sweeney *et al.*, 2007; Pradier *et al.*, 2004). Dysregulation of the lipid profile may be related, at least in part, to ART-associated effects on peripheral adipocytes resulting in increased free fatty acid (FFA) release (Mathers and Loncar, 2002; Levitt *et al.*, 2011; Mayosi *et al.*, 2009). Other consequences related to these effects include lipoatrophy, decreased cellular glucose uptake, mitochondrial damage and inhibition of hepatic TG synthesis (Flint *et al.*, 2009). ART is associated in particular with increased circulating FFA and deposition in various organs such as the liver (steatosis) and pancreas promoting insulin resistance and dyslipidaemia (Flint *et al.*, 2009). On the other hand, ART drugs such as Tenofovir have been shown to improve the lipid profile somewhat, compared to some other ARTs, such as PIs (Claas *et al.*, 2007).

##### **4.2.2.1. Total Cholesterol (TC) Levels**

Increased TC levels have previously been associated with exposure to a HFD (Tchernof and Després, 2013; Guh *et al.*, 2009); however, this was not observed in the present study

**(Fig. 3.14).** In support of our data, another study from our laboratory by Nduhirabandi *et al.* (2011; with a similar diet and feeding period), also observed no increase in mean TC levels. However, a study by Zou *et al.* (2006) found an increase in mean TC levels in the HFD group compared to the lean, control group. Zou *et al.* (2006) showed greater differences in TBM and IP fat mass between their HFD and lean control groups than in our study. Also, a greater rate in weight gain in their study compared to our study was observed. Factors that may have contributed to unchanged mean TC levels in the HFD group in our study, include duration of HFD feeding programme, degree of weight and IP fat gain and also type of fat used in the HFD (Beuttner *et al.*, 2006). The source of lipids and carbohydrates in the HFD have also been shown to variably effect TC levels in rodent models of obesity (Jurgońskie *et al.*, 2014). Jurgońskie *et al.* (2014) used a HFD that was rich in lard or soybean oil (source of saturated or unsaturated fatty acids, respectively). Each of these diets contained fructose or corn starch (source of simple or complex carbohydrates) respectively and it was found the lard- and fructose-rich diet increased serum TC and TG levels significantly, and increased the atherogenic index by more than 5-fold compared to the other diet combinations. Any of these factors and/or a combination of these factors may have contributed to the non-elevated TC level observed in our study.

Interestingly, as seen in **Table 4.2**, the TC levels were significantly higher in the ART-treated lean, control group (C/+ART) when compared to the HF/-ART and HF/+ART groups (**Fig. 3.14**). The mean TC levels in the C/+ART group was 45.79 % higher compared to the HF/+ART group. Additionally, the insulin levels (decreased vs. C/-ART and HF/+ART), and the TBARS levels (increase vs. C/-ART) levels were also affected by the ART. The increase in TC levels observed in the C/+ART compared to HF/+ART is difficult to explain, as the exact mechanism still needs to be elucidated. Treatment with NRTIs has been associated with dyslipidaemia and lipodystrophy and have been characterised by the reduction in LDL catabolism, increased LDL production, impaired FFA catabolism, increased liver TG synthesis, increased secretion of apolipoprotein-B containing lipoproteins, and reduced expression of LDL receptors as seen in **Fig. 4.1** (Freitas *et al.*, 2013).

As far as we are aware, there is no data available on the effects of this specific ART drug combination on TC levels in a rat model of obesity. However, in human studies, increased TC and LDL cholesterol levels have previously been shown to be associated with ART-treatment (Shevitz *et al.*, 2001) while higher TC levels have been associated with a greater risk for developing lipohypertrophy (Han S *et al.*, 2009). Evafirenz, one of the compounds

in the ART drug used in the current study, in particular has been shown to increase TC levels (Fontas *et al.*, 2004). The fact that TC levels were not increased in the HFD group receiving ART (HF/+ART) is difficult to explain and requires further investigation to elucidate possible mechanisms.

In summary: Although ART administration in the HFD group had no effects on the mean TC levels, the TC levels in the C/+ART group were increased compared to the HF/+ART, an interesting and novel finding which warrants further investigation.

#### 4.2.2.2. High Density Lipoprotein (HDL) Levels

HDL cholesterol is cardio-protective (and low HDL cholesterol level regarded as a CVD risk factor) (Poulter, 2003). There were no differences observed in HDL levels in any of the experimental conditions in the present study (**Fig. 4.15**) (see **Table 4.2**). However, Nduhirabandi *et al.*, 2011 (with a similar diet and feeding period), found an increase in HDL cholesterol in the HFD group. In contrast to this, a study by Zou *et al.* (2006) found a decrease in HDL cholesterol level (mg/dL) in the HFD group compared to the lean group. The reason why the HFD did not affect HDL levels in our study is unknown, but factors that may have played an important role could be the rate of weight gain, the period of feeding of the HFD and the source of lipids and carbohydrates in the HFD. All these factors have been shown to variably effect HDL levels in rodent models of obesity (Aguila *et al.*, 2002; Inai and Matsua, 2011; Jurgońskie *et al.*, 2014).

Overweight/obesity (Poulter, 2003) and increased viral load (as found in HIV-infection) have also been associated with decreased HDL cholesterol (Dubé *et al.*, 2015; Chaves *et al.*, 2003). Furthermore, long term ART, such as tenofovir (Claas *et al.*, 2007) has been associated with reduced HDL cholesterol (Hansen *et al.*, 2006; Shevitz *et al.*, 2001; Malangu N, 2014) and dyslipidaemia (characterized by high LDL cholesterol and low HDL cholesterol) (Dubé *et al.*, 2015; Chaves *et al.*, 2003; Flint *et al.*, 2009), but this was not observed in our study.

In summary: Although none of the experimental conditions had an effect on HDL cholesterol levels, a longer period of exposure to the HFD and/or ART might have had more pronounced effects on lipid regulation and result in a reduction in mean HDL cholesterol levels, as obesity and ART have previously been associated with a reduction in

HDL cholesterol. The specific ART drug combination used in the current study did not affect mean HDL cholesterol levels. This ART combination might thus only increase protective HDL cholesterol indirectly through suppressing viral replication (i.e. in the presence of HIV-infection), which was not possible to emulate in the current study.

#### 4.2.2.3. Triglyceride (TG) Levels

Studies in which animals receive a HFD usually show increased levels of circulating TGs in the body (Aguila *et al.*, 2002; Inai and Matsua, 2011; Jurgońskie *et al.*, 2014) and indeed this was also observed in the present study (see **Table 4.2**). Mean TG levels increased by 85 % in the untreated HFD group compared to the untreated lean control group, and by 40 % in the treated HFD group compared to its respective treated lean, control group (**Fig. 3.16**). The increased mean TG levels (hypertriglyceridemia) observed in the HFD groups in the present study served to further characterise and validate the HFD model, although it was not associated with insulin resistance. In terms of the effects of the HFD on mean TG levels, similar findings were observed in previous studies from our laboratory by Salie *et al.*, 2014 and Nduhiabandi *et al.*, 2014. Furthermore, Ouwens *et al.* (2005) found increased TG deposition in the heart associated with their HFD that translated into an increased heart mass similar to this study. This was further supported by a study by Zou *et al.* (2006) who found an increase in TG levels in their HFD group.

Increased circulating TG levels have previously been associated with ectopic lipid deposition in organs such as the heart (increased heart mass was found in the HFD group in the present study) (Haslam and James, 2005; Kopelman, 2007). Thus, the increased TG level may also have contributed to the increased mean heart mass (g) (C/-ART vs. HF/-ART) and also, at least in part, the increased liver mass in the HF/+ART group compared to the HF/-ART group observed in this study as discussed earlier.

As can be seen in **Table 4.2**, ART on its own had no effect on mean TG levels, suggesting that the changes observed were mainly driven by the effects of the HFD, but increased saturated fat intake in PLWH on ARTs has been noted and contributes to hypertriglyceridemia, especially among patients who have developed metabolic abnormalities such as dyslipidaemia (Joy *et al.*, 2007; Malaza *et al.*, 2012; Fedele *et al.*, 2011; Lo and Plutzky, 2012; Lo *et al.*, 2010). Although increased mean TG levels (10-12 %) have been associated with long term ART-treatment in human studies (Hansen *et al.*,

2006; Shevitz *et al.*, 2001; Flint *et al.*, 2009), the ART combination in our study had no effect on the mean TG levels, even though Evafirenz has been associated with increased TG levels previously in human studies (Fontas *et al.*, 2004).

In summary: The HFD resulted in increased TG levels in our study and possibly associated, in part at least, with increased heart mass in the HF/-ART group. The fact that TG levels were not affected by our ART drug combination in a rat model of obesity has not been described before and can therefore be considered novel; however, further investigation in this phenomenon is required.

### 4.2.3. Oxidative Stress and Lipid Peroxidation

#### 4.2.3.1. Conjugated Diene (CD) Levels

Conjugated dienes (CD) are indicators of free radical production and produced as result of unsaturated fatty acids oxidation and elevated CD levels have previously been associated with obesity (Chanta *et al.*, 2006; Clarkson *et al.*, 2000). CDs are thus good markers of early-stage lipid peroxidation (Chanta *et al.*, 2006; Clarkson *et al.*, 2000). Although obesity is associated with increased lipid peroxidation and, hence, increased CD levels (Chanta *et al.*, 2006; Clarkson *et al.*, 2000), the HFD *per se* had no effect on the mean CD levels in our study (see **Table 3.2**) (**Fig. 3.17**). Nduhirabandi *et al.* (2011) also found no difference in CD levels between control and HFD groups following the same feeding programme for the same period of time. Interestingly, the mean CD level in the HF/+ART group was 13.3 % lower compared to the HF/-ART group. The reason for this seemingly reduced oxidative stress status is unknown, as a similar effect was not observed in the TBARS levels (see later).

In summary: The HFD had no effect on the CD levels in the present study. Although overweight/obesity (Poulter, 2003) and ART (Dubé *et al.*, 2015; Hasse *et al.*, 2011; Chaves *et al.*, 2003) are known to lead to lipid-peroxidation and oxidative stress and subsequently an increase in the CD levels (Clarkson *et al.*, 2000; Matsuzawa-Nagata *et al.*, 2008), this was not observed in context of this study, but the reduction in CD level of the HF/+ART group compared to HF/-ART suggest that a possible anti-oxidant interplay exists between the HFD and ART. The results in terms of this ART combination in the

context of our study can be considered novel, as it has not been described to our knowledge.

#### 4.2.3.2. Thiobarbituric Acid Reactive Substance (TBARS) Levels

TBARS is a by-product of lipid-peroxidation (products of lipid degradation) and produced during ROS-associated cellular damage (Trevisan *et al.*, 2001; Kumar *et al.*, 2012). Because ROS has a very short half-life, measuring TBARS is a good indicator of the level of oxidative stress and lipid-peroxidation (Trevisan *et al.*, 2001; Kumar *et al.*, 2012). It is known that overweight/obesity (and associated metabolic abnormalities) can lead to oxidative stress (Lavie *et al.*, 2009; Tchernof and Després, 2013; Guh *et al.*, 2009). Also, HIV-replication (Dubé *et al.*, 2015; Breive *et al.*, 2004; Chaves *et al.*, 2003) can lead to oxidative stress and systemic inflammation (associated with CVD such as IHD (Murdoch *et al.*, 2006; Rochette *et al.*, 2013) through ROS production (Pacher *et al.*, 2005; Turko and Murad, 2002; Peluffo and Radi, 2007).

The mean TBARS levels were increased by 15.19 % in the HFD group compared to its lean control group and by 23.09 % in the lean, control animals receiving ART compared to untreated control animals (see **Table 4.2**) (**Graph 3.18**). Therefore, the HFD and ART on their own resulted in an increased mean TBARS levels and were therefore both associated with increased lipid-peroxidation and oxidative stress in our hands. In contrast to the present study, Feillet-Coudray *et al.*, 2009 found no differences in the mean TBARS levels after a high-fat, high-sucrose diet feeding programme. Nduhirabandi *et al.*, 2011 also found no difference in TBARS levels following a similar feeding programme as in our study.

In summary: The observation that the HFD resulted in increased TBARS levels further underscores the harmful physiological effects of the HFD. The increased TBARS levels observed in the ART treated lean, control group, confirmed that there was a measure of oxidative stress associated with ART as shown previously by others in human studies (Fukui *et al.*, 2001; Flint *et al.*, 2009). In our study, the ART in combination with a HFD had no effect on TBARS levels. To our knowledge the effect of ART on TBARS in a rat model of obesity has not yet been described and can be considered as novel.



### 4.3. Isolated Heart Perfusions

#### 4.3.1. Functional Recovery

The haemodynamic data obtained during the global ischaemia-reperfusion studies are summarised in **Table 4.2**.

**Table 4.3.** Summary of Haemodynamic Data – Global Ischaemia-Reperfusion (data expressed as mean  $\pm$  SEM / group (% Recovery)). **Abbreviations:** HR: Heart Rate; AO: Aortic Output; CF: Coronary Flow; CO: Total Cardiac Output; PSP: Peak Systolic Pressure; Wt: Total Power; Pre: Pre-ischaemia-reperfusion; Post: Post-Ischaemia-reperfusion. **Pre-Ischaemia vs. Post-Ischaemia:** \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ ; \*\*\*\* =  $p < 0.0001$ . (For convenience **Table 3.1** is shown here again as **Table 4.3**)

Haemodynamic Data					
Pre\$Ischaemia vs. Post\$Ischaemia					
Mean $\pm$ SEM (% Recovery)		C/+ART (Pre: N = 16; Post: N = 6)	HF/+ART (Pre: N = 14; Post: N = 6)	C/+ART (Pre: N = 11; Post: N = 4)	HF/+ART (Pre: N = 13; Post: N = 6)
HR (BPM)	Pre	224.80 $\pm$ 12.67	234.80 $\pm$ 8.72	245.90 $\pm$ 6.18	259.40 $\pm$ 17.47
	Post	205.80 $\pm$ 41.72	105.30 $\pm$ 50.13 ***	210.00 $\pm$ 23.82	201.50 $\pm$ 45.35
% Recovery		78.87 %	44.02 %	82.94 %	77.78 %
AO (ml / min)	Pre	42.09 $\pm$ 1.51	41.21 $\pm$ 1.48	42.36 $\pm$ 1.87	38.46 $\pm$ 1.57
	Post	16.58 $\pm$ 5.34 ****	7.08 $\pm$ 5.27 ****	14.50 $\pm$ 5.85 ****	7.16 $\pm$ 4.83 ****
% Recovery		39.34 %	16.99 %	32.12 %	19.16 %
CF (ml / min)	Pre	14.13 $\pm$ 0.74	14.11 $\pm$ 0.51	15.36 $\pm$ 0.74	16.08 $\pm$ 1.13
	Post	10.75 $\pm$ 2.17	6.25 $\pm$ 2.58 ***	15.75 $\pm$ 4.08	10.75 $\pm$ 2.40 *
% Recovery		76.48 %	44.12 %	93.86 %	68.16 %
CO (ml / min)	Pre	56.22 $\pm$ 1.84	55.32 $\pm$ 1.73	57.91 $\pm$ 1.81	54.54 $\pm$ 2.41
	Post	27.33 $\pm$ 7.00 ****	13.33 $\pm$ 7.56 ****	30.25 $\pm$ 9.84 ***	17.92 $\pm$ 6.10 ****
% Recovery		48.55 %	23.28 %	48.92 %	32.59 %
PSP (mmHG)	Pre	105.10 $\pm$ 1.57	100.80 $\pm$ 2.05	101.70 $\pm$ 1.69	99.15 $\pm$ 2.03
	Post	83.32 $\pm$ 16.79	41.00 $\pm$ 19.17 ****	86.0 $\pm$ 8.37	66.83 $\pm$ 16.79 *
% Recovery		77.80 %	40.94 %	82.94 %	64.44 %
Wt (mW)	Pre	13.24 $\pm$ 0.52	12.35 $\pm$ 0.43	13.24 $\pm$ 0.53	12.24 $\pm$ 0.61
	Post	6.08 $\pm$ 1.58 ****	2.44 $\pm$ 1.68 ****	6.37 $\pm$ 2.28 ***	3.56 $\pm$ 1.41 ****
% Recovery		44.90 %	19.45 %	44.21 %	27.07 %

The most important trends in terms of % recovery are discussed below:

Heart Rate (HR): Although no significant *inter*-group differences were observed in terms of % HR recovery, it was noticeable that the HFD group was the only group that presented



with a significantly lower post-ischaemia-reperfusion HR vs. pre-ischaemia-reperfusion HR values (HR recovery: %) (see **Table 4.3**).

Aortic output (AO): The AO after global ischaemia-reperfusion injury was significantly reduced compared to pre-ischaemia-reperfusion values in all the groups. Although there were no significant *inter*-group differences in the % AO recovery, it was again noticeable that the HFD groups recovered by smaller margins (range: 17 % - 19 %) compared to the lean, control hearts (range: 32 % - 39 %) (see **Table 4.3**).

Coronary flow (CF): Although no significant *inter*-group differences were observed in terms of % CF recovery, it was again observed that the hearts of the HFD groups performed poorly after the ischaemia-reperfusion insult, whereas the lean, control hearts (treated and untreated) showed better recovery rates. The addition of ART to the HFD seemed to improve the % recovery of CF when compared to the HFD without treatment (see **Table 4.3**).

Cardiac output (CO): Although, the % recovery of CO was significantly reduced in all the groups, the CO of the untreated HFD hearts (23.28 %) seemed to be lower than that of the lean control (48.55 %) and again the addition of ART to the HFD seemed to somewhat improve CO recovery to 32.55 % (see **Table 4.3**).

Peak systolic pressure (PSP): As for HR and CF, the HFD hearts (treated and untreated) were the only two groups showing significantly lower recovery in PSP. Again it seemed as though the addition of ART improved the % recovery from 41 % to 64 % (see **Table 4.3**).

Total power (Wt): Although statistically, there were no differences in the Wt recovery between the groups, it seems that the lean, control hearts performed better (range: 44 – 45 %) compared to the HFD groups (range: 19.5 – 27 %). Again we observed that the addition of ART to the HFD hearts resulted in an improved recovery (see **Table 4.3**).

From **Table 4.3** it is clear that the untreated HFD group (HF/-ART) was the only group in which ALL the recovery endpoints were significantly lower after ischaemia-reperfusion. Furthermore, the HF/-ART group was the only group in which the HR recovery was significantly poorer compared to pre-ischaemia-reperfusion (baseline) values. These trends suggest that the HFD induced myocardial changes that affected functional

performance in terms of the hearts' ability to recover from an ischaemia-reperfusion insult. These findings only apply to the recovery after the post-ischaemia-reperfusion insult, as the HFD had no effect on actual baseline values in any of the groups (see **Table 4.3**). Although assessing the effect of preconditioning in terms of a HFD (similar to the present study) vs. lean during ischaemia-reperfusion (protocol similar to the present study), Salie *et al.* (2014) found a similar trend in functional recovery (absolute values) as observed in our study when absolute values were compared within groups (Pre- vs. post-ischaemia-reperfusion: reduction in post-ischaemic recovery CF, OA, CO and Wt in the HFD group compared to HFD pre-ischaemic absolute values). Also, Nduhirabandi (2014), compared only post-ischaemia-reperfusion (absolute) values of groups with each other and, similar to our own observations, did not find differences between the lean, control and HFD groups when absolute values were compared (except for AO post-ischaemic absolute values of lean vs. HFD). In the present study, we furthermore compared the % recoveries within groups and the % recoveries between groups, but no statistical differences were observed in both comparison (see **Fig. 3.19 -24**).

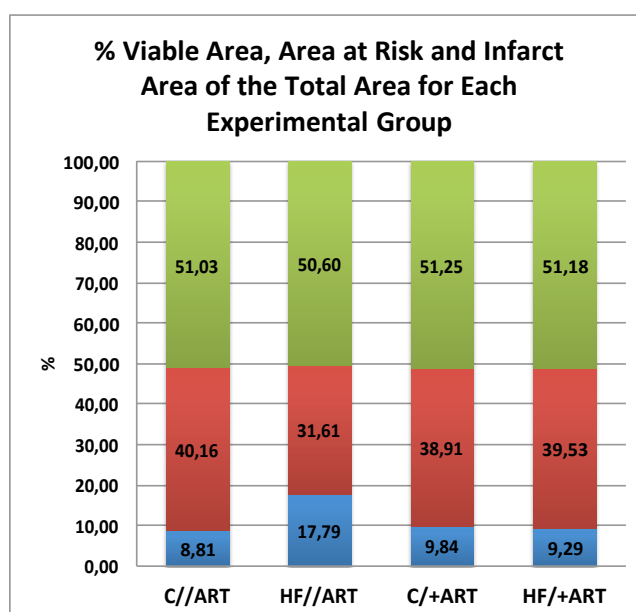
However, as mentioned earlier, some important *intra*-group observations comparing mean post-ischaemia-reperfusion values with pre-ischaemia-reperfusion values in the HFD group suggested poorer outcomes following ischaemia-reperfusion injury. It is important that our findings are seen in context. The HFD in the present study did not induce insulin resistance, as opposed to the studies by Salie *et al.* (2014) and Nduhirabandi (2014), which could explain why the poorer outcomes observed in especially the HR, CF and PSP were not sufficiently strong to translate into inter-group statistically significant differences. Small sample sizes, leading to large standard errors should also be considered. On the other hand, the HFD in the present study was associated with increased TG and lipid peroxidation (TBARS) levels, both of which are associated with myocardial dysfunction. It is therefore proposed that we observed early and moderate myocardial changes in the HFD hearts, leading to poorer recovery in all endpoints measured, the only experimental group in which this was observed.

Statistically, ART *per se* did not have any effects on any of the functional parameters assessed and as far as we are aware there are no studies that have investigated or described the effects of this specific ART drug combination nor its combination with a HFD on these parameters. Although the mean insulin level in the C/+ART group was significantly lower compared to the HF/+ART and C/-ART groups and TC was significantly elevated in the

C/+ART group compared to HF/+ART, it did not translate into myocardial dysfunction in our hands. Functionally, however, the data suggested improvements in some of the endpoints when ART was added to the HFD. Our findings in this respect can therefore be considered as novel.

#### 4.3.2. Infarct Size

There were no differences in the VA size between all groups. Also, the AR size (including the infarcted area) did not differ between groups. The infarct size (expressed as a % of AR) was greater in the HF/-ART group (35.40 %) compared to the C/-ART (18.26 %) and the HF/+ART group (19.95 %) (**Fig. 4.2**).



**Fig. 4.2.** Mean VA (green), AR (red + blue) and Infarct Area (blue) as % of Total Area after Regional Ischaemia. C/-ART: N = 8; HF/-ART: N = 7; C/+ART: N = 4; HF/+ART: N = 8.

In contrast of our findings, a study by Salie *et al.* (2014) found an infarct-sparing effect in the HFD group compared to lean, control. Bester *et al.* (2010) also found their HFD (including red-palm oil) to be infarct-sparing compared to lean, control. Obesity has previously been shown to protect the heart against ischaemia-reperfusion injury in some studies (via activation of the risk pathway) (Donner *et al.*, 2013; Salie *et al.*, 2014; Kalakech *et al.*, 2014) while others have shown a detrimental effect in terms of ischemia-

reperfusion (Aoyagi *et al.*, 2015). Similar to the findings in our study, a study by Littlejohns *et al.* (2014), found an increase in infarct size in isolated mouse hearts (N = 5-7) associated with their HFD (10-22 weeks feeding programme) compared to the lean, control group. The increased infarct size observed in the present study is supported by our functional recovery data where the HFD appeared to be more detrimental in terms of % recovery in all parameters measured. Where the addition of ART seemed to improve the functional recovery somewhat, we also observed a significant reduction in infarct size when ART is combined with the HFD.

In summary: The HFD was associated with increased infarct size during ischaemia-reperfusion in our study. On the other hand ART *per se* had no effect on infarct size. The combination of ART and HFD appeared to have infarct sparing properties. The effects of this specific ART combination in terms of isolated heart perfusions and infarct size have not been described yet and is considered novel.

#### 4.4. Western Blot Analysis

The results from Western blot analysis were obtained from hearts freeze-clamped pre- and post-ischemia. Pre-ischaemic results are summarised in **Table 4.4**, while results from post-ischaemia-reperfusion are summarised in **Table 4.5**.

**Table 4.4.** Summary of the Effects of Different Experimental Conditions on the Expression and Phosphorylation of Various Proteins of Interest (Pre-ischaemia-reperfusion). Symbols:  $\uparrow\uparrow$  = Increase;  $\downarrow\downarrow$  = Decrease; - = No Effect.

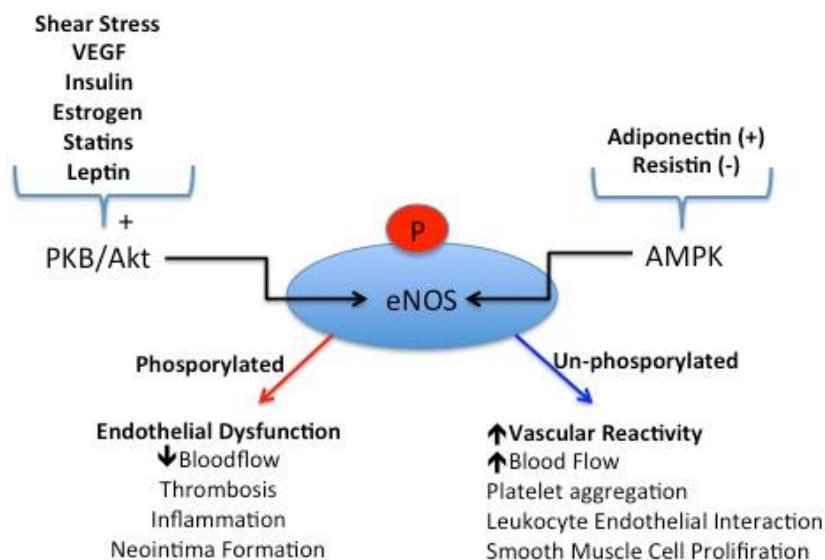
Protein	C/-ART	HF/+ART	C/+ART	HFD/+ART
NOS Signalling				
Total eNOS	-	-	!(vs. C/-ART)	!(vs. HF/-ART)
Phosphorylated eNOS	-	!(vs. C/-ART)	-	!(vs. HF/-ART)
Phosphorylated/Total eNOS	-	-	"(vs. C/-ART)	!(vs. C/+ART)
Total PKB/Akt	-	-	!(vs. C/-ART)	"(vs. C/+ART)
Phosphorylated PKB/Akt	-	-	-	"(vs. C/+ART)
Phosphorylated/Total PKB/Akt	-	-	-	-
Total AMPK	-	"(vs. C/-ART)	"(vs. C/-ART)	!(vs. HF/-ART)
Phosphorylated AMPK	-	-	-	!(vs. HF/-ART)
Phosphorylated/Total AMPK	-	!(vs. C/-ART)	!(vs. C/-ART)	-
ROS Signalling				
Nitrotyrosine	-	!(vs. C/-ART)	-	!(vs. HF/-ART)
Total P22 phox	-	!(vs. C/-ART)	!(vs. C/-ART)	!(vs. HF/-ART)
Inflammatory Signalling				
Total I $\kappa$ B $\alpha$	-	!(vs. C/-ART)	-	"(vs. HF/-ART)

**Table 4.5.** Summary of the Effects of Different Experimental Conditions on the Expression and Phosphorylation of Various Proteins of Interest (Post-ischaemia-reperfusion). **Symbols:** **Symbols:** ↑↑ = Increase; ↓↓ = Decrease; - = No Effect.

Protein	C/-ART	HF/-ART	C/+ART	HF/+ART
<b>NOS Signalling</b>				
Total eNOS	-	↓(vs. C/-ART)	-	-
Phosphorylated eNOS	-	↑(vs. C/-ART)	↑(vs. C/-ART)	↑(vs. C/+ART and HF/-ART)
Phosphorylated/Total eNOS	-	↑(vs. C/-ART)	↑(vs. C/-ART)	↑(vs. C/+ART and HF/-ART)
Total PKB/Akt	-	↓(vs. C/-ART)	↓(vs. C/-ART)	-
Phosphorylated PKB/Akt	-	↓(vs. C/-ART)	↓(vs. C/-ART)	↓(vs. C/+ART)
Phosphorylated/Total PKB/Akt	-	↓(vs. C/-ART)	-	↓(vs. C/+ART)
Total AMPK	-	↓(vs. C/-ART)	-	↓(vs. C/+ART)
Phosphorylated AMPK	-	↓(vs. C/-ART)	↓(vs. C/-ART)	↓(vs. C/+ART)
Phosphorylated/Total AMPK	-	-	↓(vs. C/-ART)	↑(vs. C/+ART)
<b>ROS Signalling</b>				
Nitrotyrosine	-	-	↑(vs. C/-ART and HF/+ART)	-
Total P22 phox	-	↓(vs. C/-ART)	-	↑(vs. C/+ART and HF/-ART)
<b>Inflammatory Signalling</b>				
Total IκBα	-	↓(vs. C/-ART)	-	↓(vs. C/-ART)

#### 4.4.1. Nitric Oxide Synthase (NOS) Signalling

eNOS, PKB/Akt and AMPK play a central role in NO production and thus cardio-protection (Huang, 2009; Chatterjee and Catravas, 2006; Simon *et al.*, 2014). Increased expression (total) and activation (phosphorylation) of these proteins in response to oxidative stress and/or inflammation (damage to the myocardium) can lead to an increase in NO production to counteract the damage and improve cardiac function (**Fig. 4.3**) (Huang, 2009; Chatterjee and Catravas, 2006; Simon *et al.*, 2014).



**Fig. 4.3.** Summary of the Role of eNOS as a Downstream Target of PKB/Akt and AMPK in the Cardiovascular System. **Abbreviations:** eNOS: Endothelial Nitric Oxide Synthase; PKB/AKT: Protein Kinase B; AMPK: 5' Adenosine Monophosphate-Activated Protein Kinase; ET-1: Endothelin-1; PI3K: Phosphatidylinositol-3-Kinase; VEGF: Vascular Endothelial Growth Factor. PKB/Akt: Protein Kinase B; P: Phosphate. **Symbols:** ↑↑ = Increase; ↓↓ = Decrease; + = Activate; - = Inhibit. (Huang, 2009; Chatterjee and Catravas, 2006; Simon *et al.*, 2014; Deng *et al.*, 2010; Dudzinski *et al.*, 2007).

These proteins interact with each other directly or indirectly causing up- or down-regulation to control NO production (Huang, 2009; Chatterjee and Catravas, 2006; Simon *et al.*, 2014). NO production inhibits apoptosis, promotes proliferation and migration of ECs, inhibits smooth muscle cell proliferation and migration (reduce thrombosis) (Napoli *et al.*, 2013; Wheeler-Jones C, 2005), inhibits platelet aggregation, adhesion of platelets, leucocytes, and monocytes to the endothelium, and reduces LDL oxidation (scavenging lipid radicals) (Lei *et al.*, 2013; Naseem, 2005; Liu *et al.*, 2008; Dias *et al.*, 2011).

#### 4.4.1.1. eNOS: Pre-Ischaemia-Reperfusion

Obesity and its associated comorbidities (diabetes, insulin resistance, hypercholesterolemia, hyperglycaemia, and hypertension) (Huang, 2009; Chatterjee and Catravas, 2006; Ceriello *et al.*, 2002) and ART (Dubé *et al.*, 2015; Hasse *et al.*, 2011)

contribute to CVD through ED and atherosclerosis and is associated with decreased NO production (Chatterjee and Catravas, 2006; Mudau *et al.*, 2012; Tabib *et al.*, 2000). In our study, the HFD had no effect on total eNOS, but the combination of HFD with ART significantly reduced total eNOS expression (see **Table 4.4**). ART itself also resulted in a 36 % reduction of total eNOS compared to lean control (see **Table 4.4**). On the other hand, the HFD resulted in a reduction in phosphorylated eNOS and when combined with ART, resulted in a further decrease (21 %) (see **Table 4.4**). When the phosphorylated eNOS was expressed as a ratio of total, ART *per se* resulted in a 42 % increase compared to lean, control and the combination of HFD and ART in a 17 % decrease compared to the HFD only (see **Table 4.4**). However, the increase in phosphorylated / total eNOS ratio by ART was likely caused by the decreased expression of eNOS, rather than increased phosphorylation. Therefore, from our results it seems that all the experimental conditions led to an overall downregulation of eNOS (despite the increased eNOS ratio in the ART treated control group). This was supported by a study by Coa *et al.*, (2011) who also found a decrease in phosphorylated / total eNOS in their HFD group compared to lean, controls.

To conclude: Obesity and its associated metabolic abnormalities such as insulin resistance and dyslipidaemia (Huang, 2009; Chatterjee and Catravas, 2006; Youn *et al.*, 2014) and ART (Dubé *et al.*, 2015; Hasse *et al.*, 2011) have been shown to contribute to CVD through ED dysfunction (*via* down-regulating eNOS (Chatterjee and Catravas, 2006) and this was indeed observed in our study as far as the HFD is concerned. The specific ART combination does not seem to contribute to CVD through eNOS in the context of our study. To our knowledge, the effects of this ART drug combination in the context of our study have not been described yet and can be considered as novel.

#### **4.4.1.2. eNOS: Post-Ischaemia-Reperfusion**

The role of NO in terms of ischaemia-reperfusion is not fully understood and studies reports on NO production in terms of cardio-protection in ischaemia-reperfusion have been inconsistent (protective, deleterious and no effects) (Flögel *et al.*, 1999; Simon *et al.*, 2014; Shulz *et al.*, 2004). Studies have shown that the administration of NO might prevent ischaemic injury *in vivo* and blood-perfused models (Flögel *et al.*, 1999; Simon *et al.*, 2014; Shulz *et al.*, 2004). The mechanism for this effect appears to involve the preservation of endothelium cell integrity and reduction of neutrophil infiltration (Flögel *et al.*, 1999;



Naseem, 2005; Simon *et al.*, 2014; Shulz *et al.*, 2004).

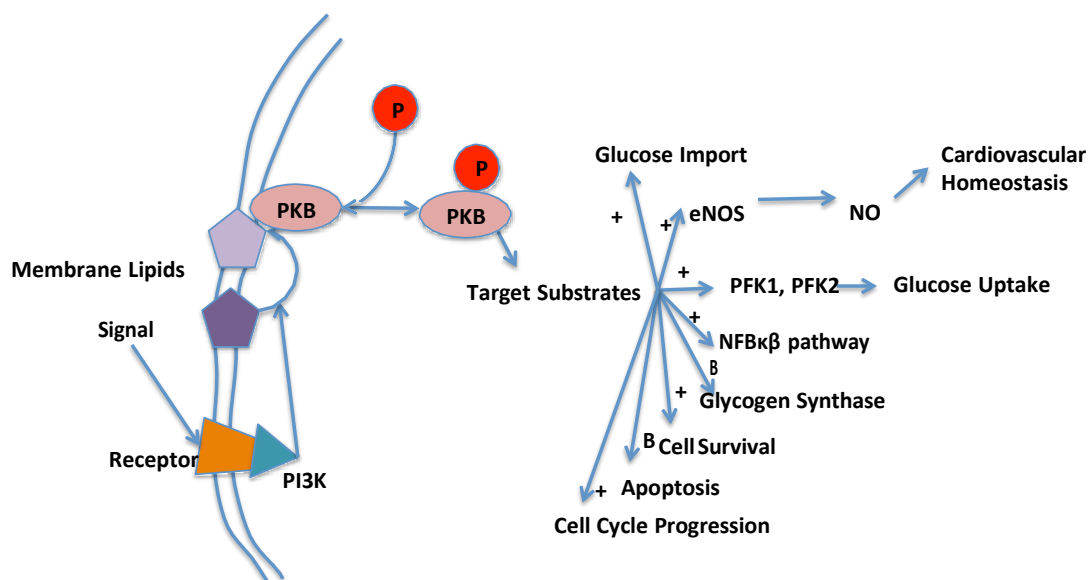
In our study, the HFD reduced total eNOS after ischaemia-reperfusion by 40 % compared to the lean controls (see **Table 4.5**). Apart from this reduction an overall increase in eNOS in all experimental conditions was seen after a global ischaemic insult (see **Table 4.5**). These trends were observed in the phosphorylation as well as the phosphorylation / total eNOS ratio in all experimental groups (see **Table 4.5**).

Although the HFD, ART and the combination of ART and HFD did not up-regulate eNOS pre-ischaemia-reperfusion, the HFD, ART and the combination of ART and obesity exhibited cardio-protective properties in terms of eNOS up-regulation (through activation as well as the ratio) post-ischaemia-reperfusion. The up-regulation of eNOS after ischaemic injury can be considered as cardio-protective, but did not appear to be sufficient to counter the reduced functional recovery and increased infarct size found in the HFD group. ART also exhibited cardio protective properties by up-regulating eNOS after ischaemia-reperfusion and even more so in combination with a HFD. This may have contributed to the improved functional recovery and smaller infarct size in the HFD combined with treatment group.

To conclude: HFD, ART and the combination of HFD and ART resulted in up-regulation of eNOS post-ischaemia-reperfusion and may have translated into cardio-protection in terms of improved functional recovery and reduced infarct-size when ART was combined with a HFD.

#### **4.4.1.3. PKB/Akt: Pre-Ischaemia-Reperfusion**

PKB/Akt is an important mediator in intracellular signal transduction (Lawlor and Alessi, 2001; Sussman *et al.*, 2011; Hemmings and Restuccia, 2012). Its activation starts with the attachment cytokines, and insulin to cell receptors that trigger the attachment of lipid kinase known as phosphoinositide 3-kinase (PI3-kinase) to the cellular membrane (**Fig. 4.4**) (Lawlor and Alessi, 2001; Sussman *et al.*, 2011; Hemmings and Restuccia, 2012).



**Fig. 4.4.** *PKB/Akt Activation and Cellular Functions.* **Abbreviations:** PKB: Protein Kinase B; PI3K: Phosphoinositide 3-kinase; P: Phosphate; eNOS: Endothelial Nitric Oxide Synthase; PFK1: Phosphofructokinase 1; PFK2: Phosphofructokinase 2; NF- $\kappa$ B: Nuclear factor kappa-light-chain-enhancer of activated B cells; NO: Nitric Oxide. **Symbols:** + = Activate; - = Inhibit. (Lawlor and Alessi, 2001; Sussman *et al.*, 2011; Hemmings and Restuccia, 2012; Morello *et al.*, 2008; Downward *et al.*, 2005; Flemming and Busse, 1999)

Activated PKB/Akt mediates downstream responses that include cell survival, growth, proliferation, migration, and angiogenesis by phosphorylating/activating various intracellular proteins (Sussman *et al.*, 2011; Hemmings and Restuccia, 2012; Shiojima and Walsh, 2006; Morello *et al.*, 2008). PKB/Akt also positively regulates eNOS and ultimately NO release (Morello *et al.*, 2008; Heusch *et al.*, 2008; Large *et al.*, 2008).

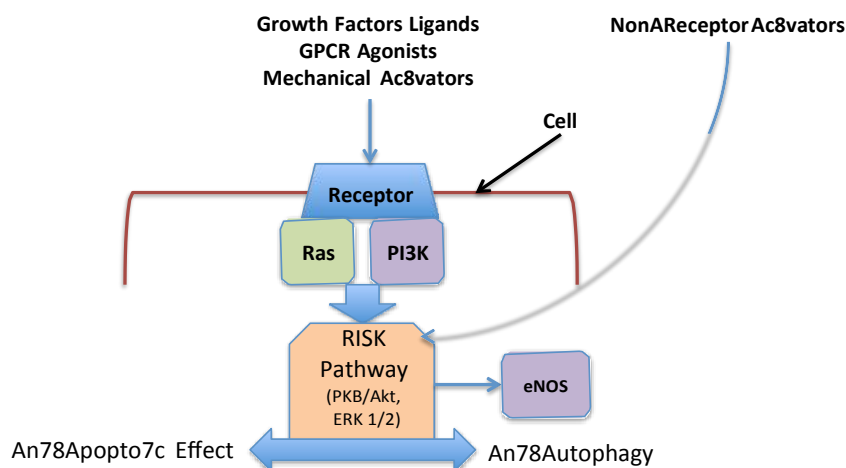
In our study, ART itself resulted in the decrease in total PKB/Akt (see **Table 4.4**). The combination of ART with a HFD however caused an increase in total and phosphorylated PKB/Akt (see **Table 4.4**). This increase was lost when expressed as a ratio (phospho / total PKB/Akt) (see **Table 4.4**). A study by Coa *et al.* (2011) found a decrease in phosphorylated PKB/Akt after their HFD programme compared to controls. However, in support of our findings, Ouwens *et al.* (2005) found similar changes in PKB/Akt expression between their lean control and HFD group, but with decreased levels of phosphorylated PKB/Akt. Down-regulation of PKB/Akt can be considered a CVD risk factor and is associated with obesity and cardiovascular risk in the heart as it can lead to decreased eNOS activity and the decreased NO production through the PKB/Akt (Huang, 2009; Simon

*et al.*, 2014; Lei *et al.*, 2013;). However, in our study, PKB/Akt was relatively unaffected in all the experimental conditions.

To conclude: Neither obesity, not ART, or the combination of obesity and ART had an effect on PKB/Akt

#### 4.4.1.4. PKB/Akt: Post-Ischaemia-Reperfusion

PKB/Akt is involved in promoting cell survival in the presence of apoptotic stimuli like ischaemia (Porstmann *et al.*, 2005; Fujio *et al.*, 2000). PKB/Akt is also involved in the regulation of nuclear factor kappa-light-chain-enhancer of activated Beta cells (NF- $\kappa$ B) and thus its expression of many pro-survival genes (Porstmann *et al.*, 2005). PKB/Akt is furthermore part of the reperfusion injury salvage kinase (RISK) pathway and form part of a group of pro-survival proteins (e.g. PKB/Akt, extracellular receptor kinase (ERK) 1 and 2 and jun N-terminal kinase (JNK) in the heart (Hausenloy and Yellon, 2007). PKB/Akt's involvement in this RISK-pathway plays a key role in cardio-protection in ischaemia-reperfusion (**Fig. 4.5**) (Hausenloy and Yellon, 2007; Yellon *et al.*, 2011).



**Fig. 4.5.** *PKB/Akt and The RISK Pathway.* **Abbreviations:** RISK: Reperfusion Injury Salvage Kinase; GPCR: G-Protein Coupled Receptor; PKB: Protein Kinase B; PI3K: Phosphoinositide 3-kinase; eNOS: Endothelial Nitric Oxide Synthase; Ras: Rat Sarcome; ERK: Extracellular Receptor Kinase. (Hausenloy and Yellon, 2007).

In our study, the HFD, ART and HFD combined with ART, resulted in an overall downregulation of the PKB/Akt signalling pathway after the global ischaemia-reperfusion insult (see **Table 4.5**). HFD as well as ART caused a reduction in total PKB/Akt. HFD, ART and the combination caused a decrease in phosphorylated PKB/Akt. All three experimental groups showed a reduction in the phosphorylated / total ratio PKB/Akt (see **Table 4.5**). Contradictory to this study, study by Salie *et al.* (2014) found no difference in PKB/Akt activation after a global ischaemic insult.

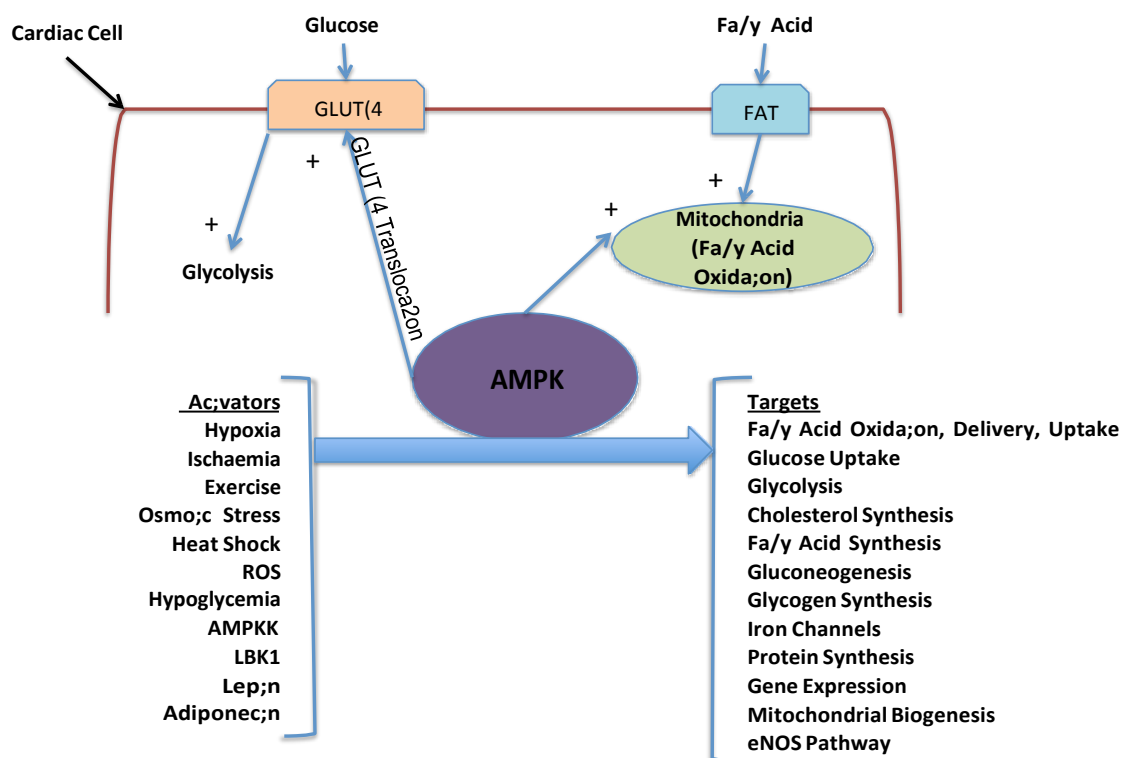
The HFD-associated down-regulation of PKB/Akt post-ischaemia-reperfusion observed in our study may have contributed to the poor functional recovery and increased infarct size also observed. Although other studies have associated obesity in rat models, with improved functional recovery and infarct-sparing properties (though activation of RISK pathway) (Salie *et al.*, 2014), this was not observed in our study.

To conclude: The HFD, ART and combination with ART in our study led to an overall down-regulation of PKB/Akt after ischaemia-reperfusion and in the case of the HFD might be in part related to the poor recovery and increased infarct size observed. Although a similar down-regulation was observed in the combination of HFD with ART, this did not completely translate to poor recovery and was not at all reflected in the reduced infarct size. Another interesting fact is that despite this overall downregulation in PKB/Akt, we still observed an overall up-regulation of eNOS after ischaemia-reperfusion, suggesting that other upstream factors may have been involved.

#### **4.4.1.5. AMPK: Pre-Ischaemia-Reperfusion**

In addition to its role as an upstream activator of eNOS, AMPK is also an energy regulating protein and is activated when ATP levels are low and regulates energy through activating energy generating pathways and inhibiting energy consumption pathways (Carling, 2004; Towler and Hardie, 2007; Heidrich *et al.*, 2010). Activated AMPK furthermore plays a further role in regulating insulin secretion and gene expression in the pancreatic  $\beta$  cells, while insulin in turn inhibits AMPK (e.g. in the heart), suggesting a complex interplay (Carling, 2004; Carling, 2005; Kahn *et al.*, 2005). AMPK is activated during obesity due to several factors that include increased obesity-associated energy demand, hypoglycaemia, associated increased haemodynamic load and associated metabolic stress (Dyck, 2006). Glycolysis is also directly stimulated by AMPK by directly

activating phosphofructokinases while activated AMPK decreases glycogen synthase activity (Arad *et al.*, 2007). Activated AMPK can have many downstream consequences in cells as shown in **Fig. 4.6**.



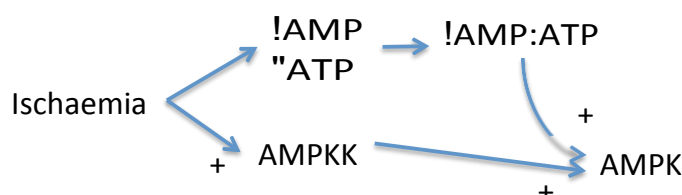
**Fig. 4.6.** An Overview of AMPK Activation and Role in Cardiac Cells. **Abbreviations:** LKB1: Liver Kinase-1; GLUT-4: Glucose Transporter-4; ROS: Reactive Oxygen Species; AMPKK: AMPK Kinase; FAT: FA Transporter; eNOS: Endothelial Nitric Oxide Synthase. **Symbols:** + = Activate (Miller *et al.*, 2005; Heinrich *et al.*, 2010; Minokishi *et al.*, 2002; Nagendran *et al.*, 2013).

In our study, only the combination of the HFD with ART seemed to decrease both total and phosphorylated eNOS (see **Table 4.4**). The reductions observed in the HFD and the ART groups (compared to lean control), could be due to the increase found in total AMPK and no change in phosphorylated (activated) AMPK. Therefore these two experimental conditions did not seem to have an effect on AMPK pre-ischaemia-reperfusion. A study by Pang *et al.*, 2008 found a decrease in AMPK in their HFD group. Contradictory to this study, a study by Coa *et al.* (2011) found an increase in phosphorylated AMPK in their HFD group.

To conclude: Only the combination of a HFD with ART seemed to have an effect (decrease) in AMPK (total and phosphorylated), although this was not the case when phosphorylated AMPK was expressed as a ratio of total AMPK.

#### 4.4.1.6. AMPK: Post-Ischaemia-Reperfusion

Ischaemia decreases ATP concentration (due to hypoxia) in the cardiac cells and increases cellular glucose uptake in the heart through GLUT-4 to increase ATP production for continued contractility of the heart muscle (**Fig. 4.7**) (Miller *et al.*, 2005; Paiva *et al.*, 2010; Heinrich *et al.*, 2010).



**Fig. 4.7.** AMPK Activation During Ischaemia. **Abbreviations:** AMP: Adenosine Mono Phosphate; ATP: Adenosine Triphosphate; AMPKK: AMPK Kinase. **Symbols:** + = Activate; ↑↑ = Increase; ↓↓ = Decrease (Miller *et al.*, 2005; Paiva *et al.*, 2010).

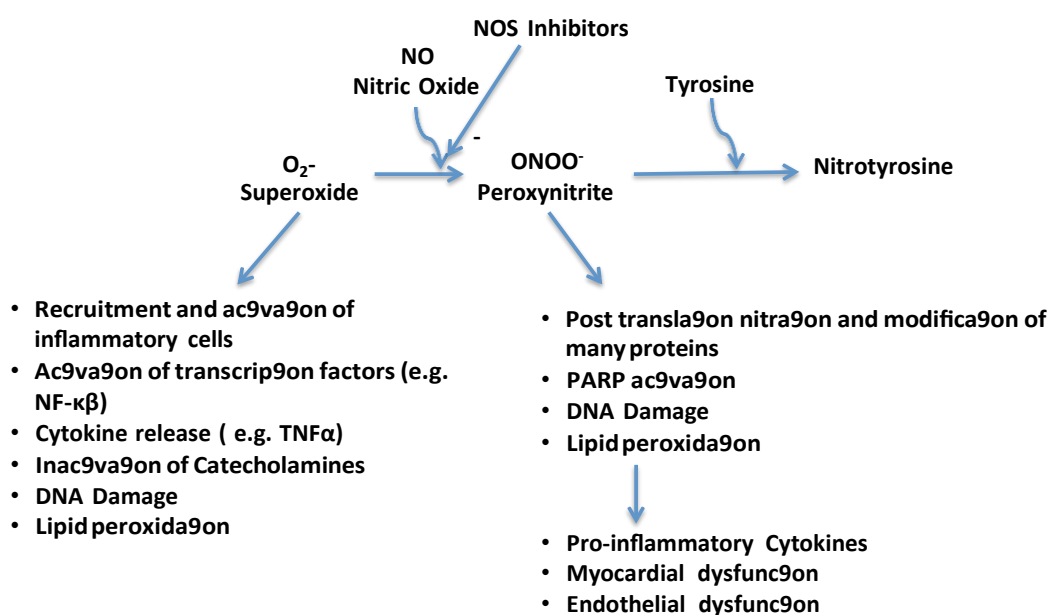
Activated AMPK during ischaemic stress leads to increased glucose uptake, glycolysis and FA oxidation that subsequently lead to cardio-protection through preventing apoptosis (Miller *et al.*, 2005; Paiva *et al.*, 2010).

In our study, all three experimental conditions seemed to cause an overall downregulation of AMPK (see **Table 4.5**). This reduction observed in the phosphorylated / total AMPK ratio is in agreement with the findings of a study by Pang *et al.*, 2008. Contradictory to this study, a study by Coa *et al.* (2011) found an increase in phosphorylated AMPK in their HFD group.

To conclude: All of the experimental conditions (with the exception of the increased phosphorylated / total AMPK in the HFD + ART group), seemed to downregulate AMPK. Again this was somewhat surprising as despite the overall downregulation of both PKB/Akt and AMPK (upstream activators of eNOS), we still found an overall increase in eNOS.

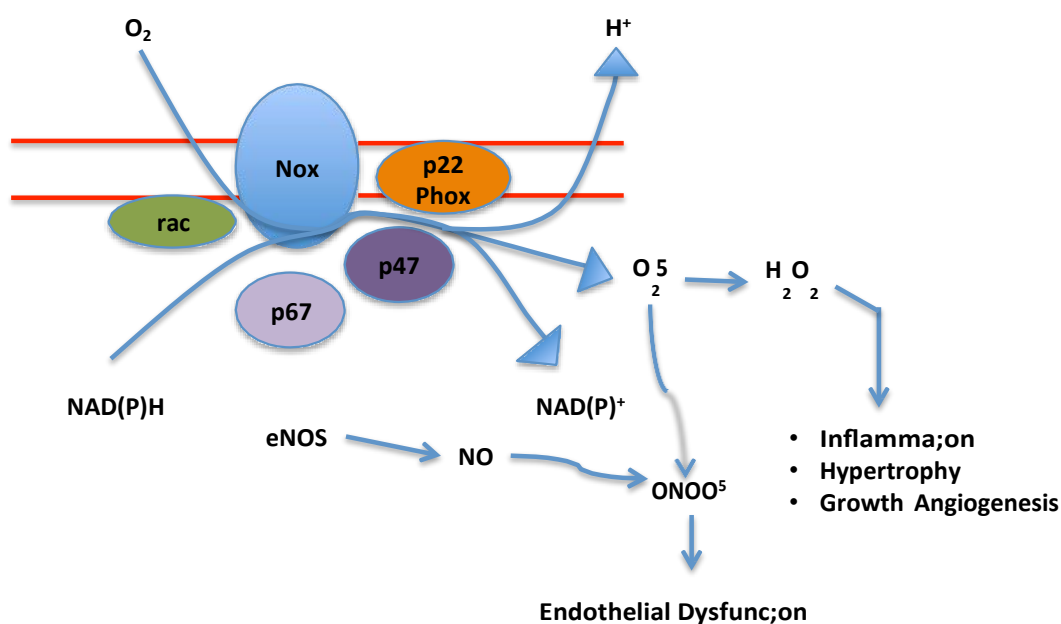
#### 4.4.2. Proteins Associated with Oxidative Stress

Pathophysiological conditions such as oxidative stress increases the production of superoxide ( $O_2^-$ ) and NO (Strijdom, 2012; Shishehbor *et al.*, 2003; Pacher *et al.*, 2007). A diffusion-controlled reaction (spontaneous) between NO with  $O_2^-$  leads to the formation of peroxynitrite ( $ONOO^-$ ) (Strijdom, 2012; Shishehbor *et al.*, 2003; Turko and Murad, 2002; Peluffo and Radi, 2007). Increased generation of ROS, such as superoxide ( $O_2^-$ ), result in oxidative stress, which is harmful to the myocardium (Strijdom, 2012; Pacher *et al.*, 2007; Peluffo and Radi, 2007). Nitrotyrosine is a by-product in the production of the harmful radical, peroxynitrite ( $ONOO^-$ ), and thus a good marker of  $ONOO^-$  (Strijdom, 2012; Pacher *et al.*, 2007; Peluffo and Radi, 2007).  $ONOO^-$  in turn is a destructive free radical oxidant that can damage DNA, membrane lipids, and mitochondria and can result in cellular apoptosis and necrosis (**Fig. 4.8**) (Strijdom, 2012; Shishehbor *et al.*, 2003; Pacher *et al.*, 2007).



**Fig. 4.8.** Nitrotyrosine as a Marker for Peroxynitrite and Ultimate ED and CV Dysfunction. **Abbreviations:** NO: Nitric Oxide; NOS: Nitric Oxide Synthase; NF- $\kappa$ B: Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells; TNF- $\alpha$ : Tumor Necrosis Factor Alpha; DNA: Deoxyribonucleic Acid; PARP: Poly ADP Ribose Polymerase. **Symbols:** - =Inhibit. (Strijdom, 2012; Shishehbor *et al.*, 2003; Pacher *et al.*, 2007; Turko and Murad, 2002; Peluffo and Radi, 2007; Buonocore *et al.*, 2010; Ray *et al.*, 2005).

P22 phox is one of several subunits of the  $O_2^-$  generating NADPH oxidase and thus a good marker of NADPH-oxidase activation and hence NADPH-oxidase dependent  $O_2^-$  production (**Fig. 4.9**) (San Jose *et al.*, 2008; Griending *et al.*, 2000; Cahilly *et al.*, 2000).

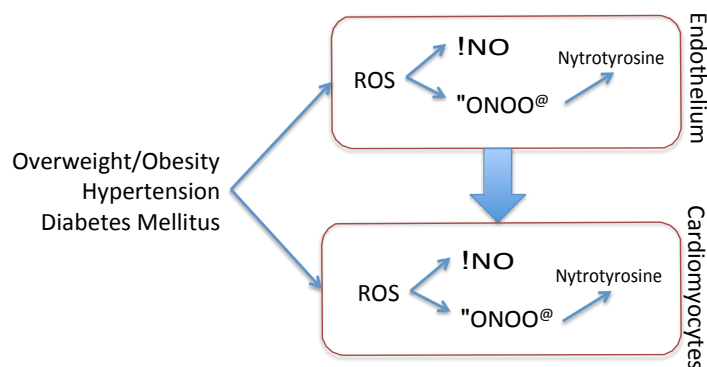


**Fig. 4.9.** Schematic Illustration of Vascular NADPH Oxidase Activation and Its Component P22 Phox. **Abbreviations:** Nox: Vascular NADPH Oxidase; rac: Ras-Related C3 Botulinum Toxin; P22 Phox, p47, p67: Subunits of the NADPH-Oxidase Complex. NO: Nitric Oxide. eNOS: Endothelial Nitric Oxide Synthase; NADPH: Nicotinamide Adenine Dinucleotide Phosphate (San Jose *et al.*, 2008; Griending *et al.*, 2000; Cahilly *et al.*, 2000; Bedard and Krause, 2007).

#### 4.4.2.1 Nitrotyrosine: Pre-Ischaemia-Reperfusion

Obesity and its metabolic abnormalities such as insulin resistance, hyperglycaemia and hypercholesterolemia are known to cause nitrosative and oxidative stress in the heart (increase in ROSs such as  $O_2^-$  and  $ONOO^-$ ) and contribute to CVD (**Fig. 4.10**) (Pacher *et al.*, 2005; Hensley *et al.*, 2000; Csaba *et al.*, 2007).





**Fig. 4.10. Nitrotyrosine and Oxidative Stress in Obesity.** **Abbreviation:** ROS: Reactive Oxygen Species; NO: Nitric Oxide. **Symbols:**  $\uparrow\uparrow$  = Increase;  $\downarrow\downarrow$  = Decrease; (Pacher *et al.*, 2005; Hensley *et al.*, 2000).

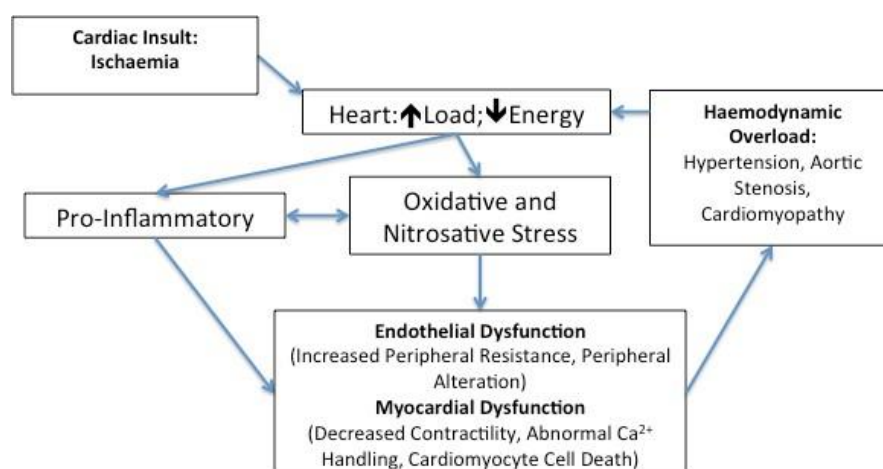
In our study, the HFD and when combined with ART led to decreased nitrotyrosine (see **Table 4.4**) pre-ischaemia. In contrast to our study, Rennison and Wagoner (2009) showed increased nitrotyrosine levels in a HFD rat model, but Relling *et al.* (2006) did not find any changes in nitrotyrosine levels after their HFD rat model. Although obesity has been associated with increased oxidative stress (nitrotyrosine level is an indicator of oxidative stress) due to the production of ROS (such as peroxynitrite) in the vasculature and has been associated with atherosclerosis and other CVDs (Pacher *et al.*, 2005; Hensley *et al.*, 2000; San Jose *et al.*, 2008), the opposite was found in our study. Interestingly, the combination of ART and HFD resulted in even lower nitrotyrosine levels.

To conclude: Surprisingly, obesity reduced nitrotyrosine levels pre-ischaemia-reperfusion and induced even a further reduction when combined with ART, although ART *per se* did not affect nitrotyrosine levels. These findings in terms of ART, has not been described yet and can thus be considered as novel.

#### 4.4.2.2. Nitrotyrosine: Post-Ischaemia-Reperfusion

During global and regional ischaemia oxidative and nitrosative stress is increases (Pacher *et al.*, 2005; Hensley *et al.*, 2000; Yasmin *et al.*, 1997).  $\text{OONO}^-$  is rapidly produced during acute reperfusion of ischaemic hearts and leads to the formation of nitrotyrosine (**Fig. 4.11**) (Ferdinandy and Schulz, 2003; Ferdinandey P, 2015). This can lead to decreased

left ventricular function, increased myocardial damage, apoptosis, increased neutrophil infiltration, increased circulating tumor necrosis factor alpha (TNF- $\alpha$ ), and increased infarct size (Pacher *et al.*, 2005; Hensley *et al.*, 2000).



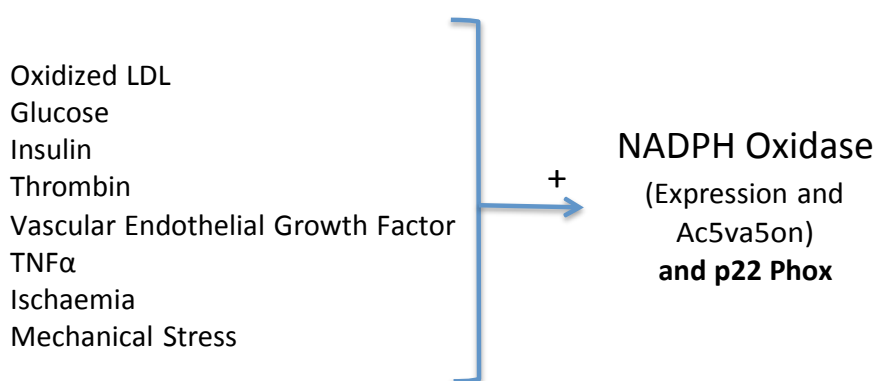
**Fig. 4.11.** Nitrotyrosine and Oxidative Stress in Ischaemia-Reperfusion. Symbols: ↑↑ = Increase; ↓↓ = Decrease (Pacher *et al.*, 2005; Hensley *et al.*, 2000).

In our study, only ART resulted in a 35 % increase in nitrotyrosine compared to lean, control as well as HFD with ART (see **Table 4.5**). Although obesity has been associated with increased nitrotyrosine production post ischaemia (Pacher *et al.*, 2005; Hensley *et al.*, 2000), this was not observed in our study. ART increased post-ischaemic nitrotyrosine levels. This may be related to the ART-associated AMPK and PKB/Akt down-regulation we also observed in post-ischaemia-reperfusion in our study, but needs further investigation.

To conclude: In the present study, ART was associated with increased nitrotyrosine levels compared to lean control in the post-ischaemia-reperfusion setting. This is consistent with findings in human studies that also associated ART with increased oxidative stress, but interestingly obesity ameliorated this. These findings in terms of ART is considered novel, as it has not been described yet, to our knowledge.

#### 4.4.2.3. P 22 Phox: Pre-Ischaemia-Reperfusion

Obesity, insulin resistance and hyperlipidaemia have been associated with oxidative stress through NO dysregulation and increased ROS generation due to decreased NOS bioavailability that leads to CVD (Roberts *et al.*, 2006; Shulman GI, 2000). ROS may increase expression and phosphorylation of NADPH-oxidase (Murdoch *et al.*, 2006). NADPH oxidase can also be activated and expressed via numerous other factors (**Fig. 4.12**) (Murdoch *et al.*, 2006).



**Fig. 4.12.** Factors that Lead to Increased NADPH Oxidase Activation and Expression and its Component P22 Phox. **Abbreviations:** P22 Phox: Subunits of the NADPH-Oxidase Complex. NADPH: Nicotinamide Adenine Dinucleotide Phosphate; LDL: Low-Density Lipoprotein; TNF-α: Tumor Necrosis Factor Alpha. **Symbols:** + = Activation (Murdoch *et al.*, 2006; Heymes *et al.*, 2003; Jones *et al.*, 2005).

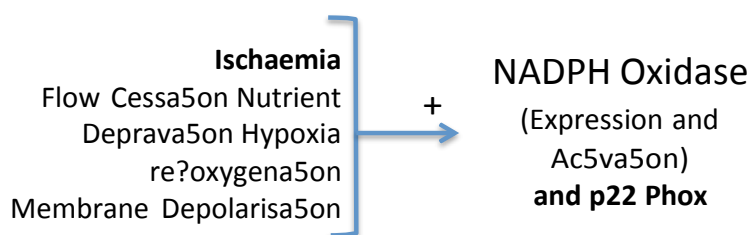
Interestingly, a decrease in p22 phox expression was observed in all the experimental conditions compared to the untreated lean control group pre-ischaemia-reperfusion (see **Table 4.4**). Contrary to our findings, Feillet-Coudray *et al.* (2009) found an increase in NADPH activity in their HFD group. Also, Liu J and Lloyd (2013) found increased NADPH activity following their HFD. The decreased level of p22 phox is, amongst others, an indicator of decreased NADPH-oxidase induced ROS production and could reflect a state of reduced oxidative stress, which is cardio-protective (San Jose *et al.*, 2008; Griendling *et al.*, 2000; Bedard and Krause, 2007; Cai, 2005). Although obesity has previously been shown to be associated with increased p22 phox levels (Murdoch *et al.*, 2006; Roberts *et al.*, 2006), this was not observed in the current study. On the other hand, ART has also previously been associated with oxidative stress (Dubé *et al.*, 2015; Hasse *et al.*, 2011;

Chaves *et al.*, 2003) in human studies, but in terms of p22phox and NADPH oxidase signalling, this ART FDC appeared to be beneficial as it reduced ROS production (even more so in combination with a HFD). The decrease in p22 phox levels is also in contrast to increased TBARS levels observed in the HF/-ART group compared to the lean, control, suggesting another protective mechanism may have been activated in the heart against obesity-associated systemic oxidative stress. The reason for the decreased p22 phox level in lean, treated group compared to the lean, control may indicate that this specific ART combination might be beneficial in protecting the heart against oxidative stress although this was not observed in terms of nitrotyrosine.

To conclude: The HFD, ART and the combination of HFD with ART seemed to be cardio-protective in terms of p22 phox-associated oxidative stress. These ART related findings is considered novel as the effects of this ART combination in the context of our study has not yet been described.

#### 4.4.2.4. P22 Phox: Post-Ischaemia-Reperfusion

Numerous factors during ischaemia contribute to the activation of NADPH-oxidase and its p22 phox subunit (**Fig. 4.13**) (Murdoch *et al.*, 2006; Kimura *et al.*, 2005).



**Fig. 4.13.** Factors that Lead to Increased NADPH Oxidase and Its Component P22 Phox Activation and Expression During Ischaemia-Reperfusion. **Abbreviations:** P22 Phox: Subunits of the NADPH-Oxidase Complex. NADPH: Nicotinamide Adenine Dinucleotide Phosphate (Murdoch *et al.*, 2006; Yoshikawa and Abe, 2001).

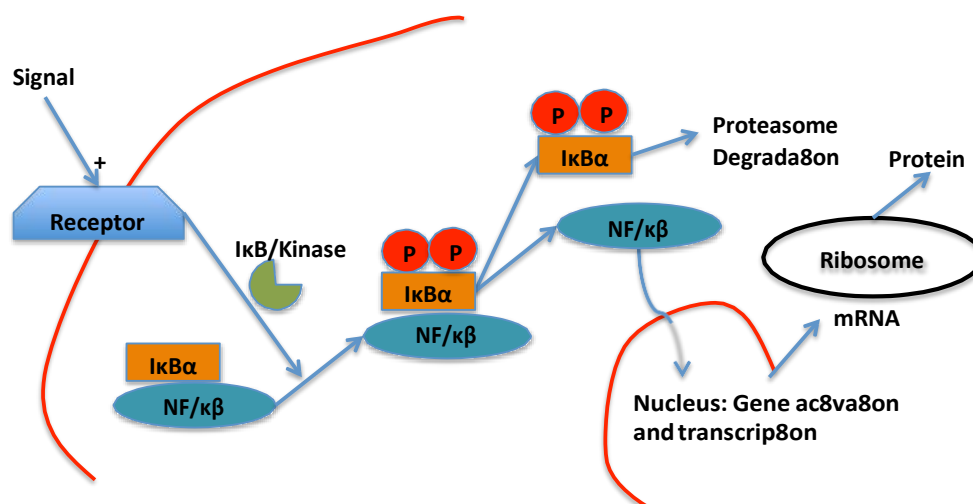
P22 phox, TBARS, and NF-kB levels have been associated with increased infarction after MI (Fukui *et al.* (2001) Fukui *et al.*, 2004). In our study, the HFD reduced p22 phox levels

compared to lean control (32 %) post ischaemia-reperfusion. Although ART itself did not affect p22 phox levels in our study, the combination of ART and obesity increased p22 phox by 50 % compared to lean, treated and 65 % compared to the HFD itself (see **Table 4.5**). These results indicate that the HFD reduced oxidative stress and the reason for this may be due to the HFD-associated up-regulation of eNOS observed after ischaemia-reperfusion in our study. This reduction in oxidative stress did not translate in improved functional recovery as infarct size was increased and functional recovery reduced compared to all experimental conditions.

To conclude: The HFD in our study resulted in decreased levels of p22 phox and thus reduced oxidative stress compared to lean, controls (not reflected in poor functional recovery and increased infarct size). When the HFD was combined with ART, oxidative stress (in terms of p22 phox) was increased. Findings in terms of ART have not been describe in the context of your study and was considered novel.

#### 4.4.3. Pro-Inflammatory NF- $\kappa$ B Signalling

NF- $\kappa$ B activates the transcription of many genes associated with inflammation (Jones *et al.*, 2005; Brasier, 2006; Pierce *et al.*, 2009). I $\kappa$ B $\alpha$  regulates the transcription factor NF- $\kappa$ B by keeping it in an inactive state and thus inhibiting inflammatory signalling (Huxford and Ghosh, 2009; Jacobs and Harrison, 1998; Baeuerle, 1998) (**Fig. 4.14**).

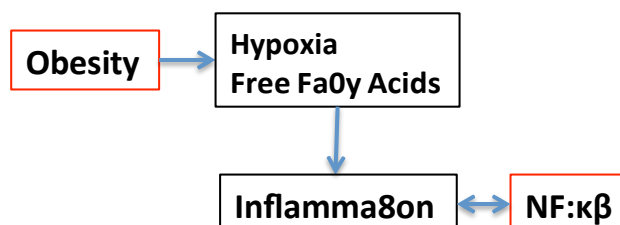


**Fig. 4.14.** *I $\kappa$ B $\alpha$  Inhibition of NF- $\kappa$ B.* **Abbreviations:** I $\kappa$ B $\alpha$ : Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B-cells Inhibitor, Alpha; NF- $\kappa$ B: Nuclear factor kappa-light-chain-enhancer of activated B cells; I $\kappa$ B-Kinase: Multi-kinase complex; P: Phosphate. **Symbols:** + = Activate. (Huxford and Ghosh, 2009; Jacobs and Harrison, 1998; Baeuerle, 1998)

In the CV system, NF- $\kappa$ B activation is associated with myocarditis, ischaemic injury, MI, dilatation, heart failure, cardiac hypertrophy, atherosclerosis and ED (Jones *et al.*, 2005). I $\kappa$ B $\alpha$  inhibition of NF- $\kappa$ B plays thus an important role in CV function (Jones *et al.*, 2005).

#### 4.4.3.1. I $\kappa$ B $\alpha$ : Pre-Ischaemia-Reperfusion

During obesity, NF- $\kappa$ B expression is increased and positively related to nitrotyrosine (Pierce *et al.*, 2009). Studies further found that vascular ED in obesity is in part mediated via the NF- $\kappa$ B pathway (**Fig. 4.15**) (Pierce *et al.*, 2009).



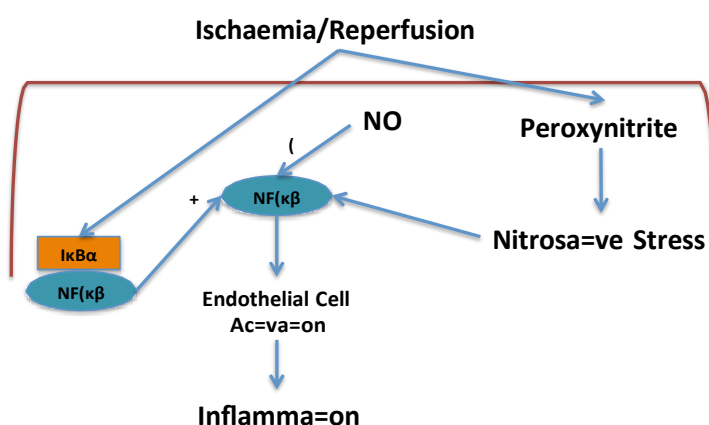
**Fig. 4.15.** Obesity Leading to Inflammation and NF- $\kappa$ B Activation. **Abbreviations:** I $\kappa$ B $\alpha$ : Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B-cells Inhibitor, Alpha; NF- $\kappa$ B: Nuclear Factor Kappa-Light-Chain-Enhancer of Activated Beta cells (Pierce *et al.*, 2009).

In our study, the HFD caused a 14 % reduction in I $\kappa$ B $\alpha$  expression (compared to lean, controls), while the combination of the HFD with ART resulted in an increase of 28 % in I $\kappa$ B $\alpha$  levels (see **Table 4.4**). These findings suggest that the HFD induced activation of the pro-inflammatory NF- $\kappa$ B pathway and reduced NF- $\kappa$ B activation when combined with ART. Obesity (Pierce *et al.*, 2009) and ART-treatment *per se* (Dubé *et al.*, 2015; Hasse *et al.*, 2011; Chaves *et al.*, 2003) have previously been associated with inflammation and this was reflected in our finding in terms of our HFD. The HFD in combination with ART exhibited anti-oxidative stress properties (decreased nitrotyrosine and p22 phox levels) and this was indeed reflected in decreased inflammatory signalling.

To conclude: The HFD in our study seemed to induce pro-inflammatory signalling, while when combined with ART it seemed to result in a reduction. This is possibly due to the anti-oxidative stress properties observed. The effect of this ART combination in the context of our study has not been described yet, to our knowledge, and is considered novel.

#### 4.4.3.2. $\text{I}\kappa\text{B}\alpha$ : Post-Ischaemia-Reperfusion

Ischaemia-reperfusion produces ROS (e.g. NO) and reactive nitrogen species such as peroxynitrite that's involved in cell death (Jones *et al.*, 2005). There is some controversy over whether  $\text{NF-}\kappa\text{B}$  is cardio-protective or cardio-damaging because of its role in activation pro-cell survival genes after hypoxia and ischaemia preconditioning also, but has been found to be predominantly cardio-damaging after ischaemia-reperfusion (Jones *et al.*, 2005; Csonka *et al.*, 2001; Downey *et al.*, 2007). Nonetheless,  $\text{NF-}\kappa\text{B}$  is a powerful activator of pro-inflammatory cytokines and an activator of pro-apoptotic genes during ischaemia-reperfusion, which makes its inhibitor in response to inflammation in cardiac cells a powerful indicator of inflammation during ischaemia-reperfusion (**Fig. 4.16**) (Jones *et al.*, 2005).



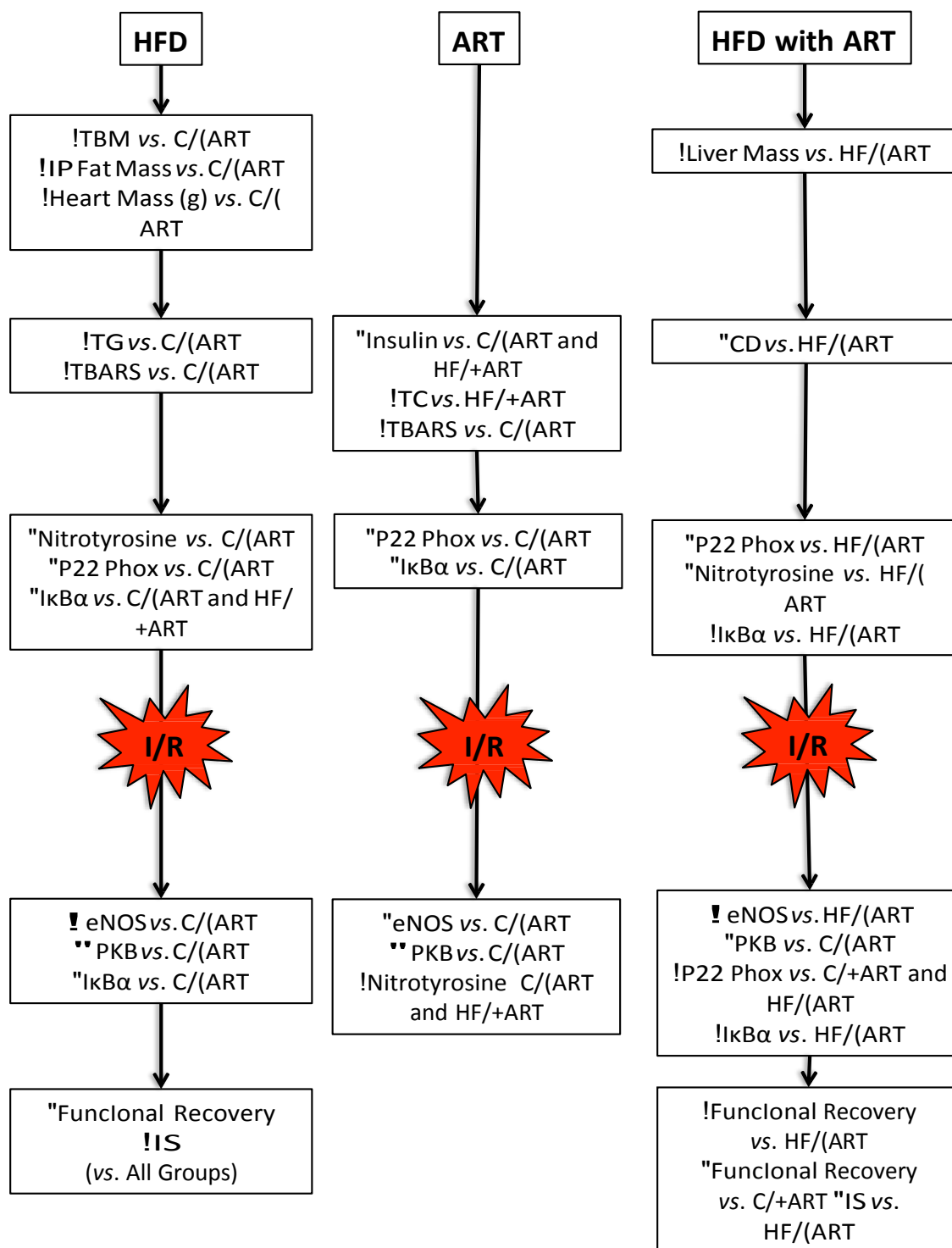
**Fig. 4.16.** *IκBα* Dissociates from  $\text{NF-}\kappa\text{B}$  During Ischaemia-Reperfusion Leading to Inflammation. **Abbreviations:** NO: Nitric Oxide;  $\text{I}\kappa\text{B}\alpha$ : Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B-Cells Inhibitor, Alpha;  $\text{NF-}\kappa\text{B}$ : Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells. NO: Nitric Oxide; **Symbols:** + = Activate; - = Inhibit (Jones *et al.*, 2005).

In our study, both the HFD and when combined with ART reduced  $\text{I}\kappa\text{B}\alpha$  levels after ischaemia-reperfusion (see **Table 4.5**). Thus inflammatory signalling was increased by both the HFD and the combination of the HFD with ART in our study after an ischaemic insult. These findings do not correlate with the reduced oxidative stress and overall up-regulation we observed in these groups post-ischaemia and needs further investigation.



In summary: In our study, both the HFD and its combination with ART led to increased pro-inflammatory NF- $\kappa$ B signalling, ART did not have any effect on inflammatory signalling post-ischaemia-reperfusion. To our knowledge, the effects of this specific ART combination post-ischaemia-reperfusion in the context of this study had not been described yet and is thus considered novel.

#### 4.5. Summary of the Main Findings



**Fig. 4.17.** Summary of Results. **Abbreviations:** IS: Infarct Size; I/R: Ischaemia-Reperfusion **Symbols:** ↑↑ = Increase; ↓↓ = Decrease.

The HFD in our study was successful in inducing increased body weight, IP fat mass and heart mass (g). This was further validated by increased TG levels. Cardiovascular risk associated with a HFD was underscored in our study in terms of relatively poorer functional recovery and increased infarct size after an ischaemia-reperfusion insult. The HFD possibly contributed to decreased functional recovery and increased infarct size though increased TBM, increased IP fat mass, increased heart mass, increased TG and TBARS levels, down-regulation of AMPK, up-regulation of inflammatory signalling and down-regulation of PKB/Akt. The improvement observed when ART was combined with a HFD (especially in terms of infarct size and some of the functional recovery endpoints) was possibly due to reduced oxidative stress (reduced CD levels, reduced nitrotyrosine levels and reduced p22 phox expression) and an up-regulation of eNOS. Although there was an increase in TBARS levels, it was not supported by the reduction found in nitrotyrosine and p22 phox levels.

ART on its own led to an increase in TC and TBARS levels as well as an increase in nitrotyrosine after ischaemia. However, on the other hand, it seemed to induce the cardio-protective eNOS pathway after an ischaemic insult. Apart from these findings, this specific ART drug combination did not seem to have a profound effect on any of the other parameters assessed. When the HFD was combined with ART, the ART seemed to ameliorate the harmful effects caused by the HFD itself, particularly in terms of the isolated heart perfusion studies, as was seen in the slight improvement in functional recovery as well as the reduced infarct size.

## Chapter 5 – Final Conclusion

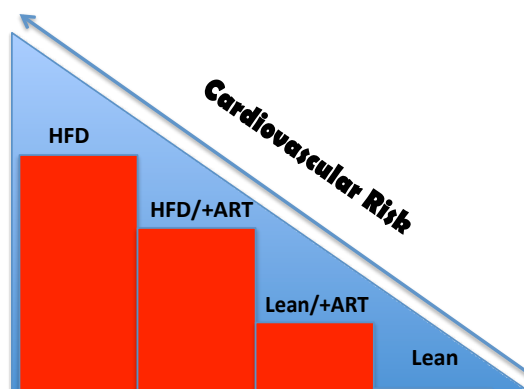
### 5.1. Conclusion

In the present study the HFD resulted in increased the TBM, IP fat mass and heart mass. Despite increasing the TG levels, the HFD was unsuccessful in inducing insulin resistance. Although the HFD increased oxidative stress as demonstrated by elevated TBARS levels, the other markers of oxidative stress (nitrotyrosine and p22 phox levels) were decreased both in the pre-and post-ischaemia-reperfusion scenario. Overall, ALL endpoints measured as markers of myocardial function in the HFD group were significantly and negatively affected by the ischaemia-reperfusion insult, something which was not observed in any of the other groups, and this was associated with an increased infarct size following regional ischaemia-reperfusion. Although eNOS was upregulated (only after ischaemia-reperfusion) in the HFD, PKB/Akt (RISK pathway component) was downregulated. The overall downregulation in PKB/Akt signalling might explain the poor recovery and increased infarct size that we observed. There was also an increase in pro-inflammatory NF- $\kappa$ B activation.

Although ART in lean animals led to increased TC levels, it had no effect on TBM, IP fat and heart mass. ART led to increased TBARS levels, yet this was accompanied by decreased nitrotyrosine and p22 phox levels. Oxidative stress was evident in lean animals receiving ART after the global ischaemia-reperfusion insult as demonstrated by increased nitrotyrosine levels. Apart from the TBARS and increase in nitrotyrosine (post-ischaemia), ART did not seem to have any effect on any of the other parameters assessed.

ART combined with a HFD exerted interesting effects. Although the increases in TBM and IP fat mass were not affected by ART, it did additionally result in liver mass in the HFD animals. The administration of ART to the HFD animals seemed to alleviate some of the harmful effects we observed in the HFD only. This could be observed in the overall upregulation of the cardioprotective eNOS enzyme, which was associated with a modest improvement functional recovery and a significant reduction in infarct size (when compared to HFD only).

This study thus underscores the detrimental effect of a HFD and obesity on cardiovascular health compared to lean, control. Interestingly, when combined with ART, there seemed to be an improvement in some endpoints of cardiovascular health compared to the HFD itself. ART administration to lean animals exhibited little effect on cardiovascular health in our study compared to lean, control, but improved when compared to in combination with a HFD (**Fig. 5.1**).



**Fig. 5.1.** *Suggested Overall Cardiovascular Risk Associated with Each Experimental Condition.*

Findings regarding this specific ART combination in terms of cardiovascular health parameters assessed in a rat model of obesity are considered novel, as it has not been described yet in the context of the present study.

## 5.2. Shortcomings

Although the HFD model in this study has previously been established, assessing the effect of this particular ART combination in a rat model of obesity was novel. No information from previous studies could be used for our study design or to improve on previous studies. Little is known on the effect of ART *per se*, in the context of obesity on cardiovascular health. Parameters assessed in our study had to be carefully selected on limited information available from human studies mostly. As little is known about the overlap of obesity and ART in terms of cardiovascular risk/disease, selecting parameters to be assessed during our study, relied mostly on suggestions from literature (human

studies) in terms of some parameters that might lay at the intersection of obesity, ART and cardiovascular disease.

The number of animals we could assign to each experimental group was also a limitation in our study. This was mainly due to the cost per animal on a 16-week diet program in the animal facilities as well as the time restraints in completing experimentation after a lengthy feeding programme. It was also originally decided to administer the drug combination in jelly cubes as has previously been used for other drug-studies in our laboratory. However, the animals refrained from eating the jelly cubes and we had to change our protocol from the above to gavaging of the animals to ensure daily administration of the drug. This resulted in the loss of animals and reduced our sample size per group for all parameters assessed. Some of these experimental procedures in our study require a larger than usual sample size as the data points do not tend to cluster. Greater sample sizes may have improved accuracy and elucidated borderline significant differences between experimental groups.

Also, the cardiovascular effects of this ART combination may have become more pronounced if the 16-week feeding programme and/or the 6-week drug treatment programme were extended beyond 16 and 6 weeks. As HIV-populations are aging and becoming more drug-experienced, studying the long-term effects of ART is becoming more relevant (also in terms of obesity).

Furthermore, our HFD did not succeed in inducing insulin resistance in the HFD groups. This may have implications on parameters assessed in our study.

It would have been ideal to investigate the effects of ART in an animal model of HIV and obesity combined, but this was not feasible.

### **5.3. Future Directions**

Our study only focused on a few parameters that can be affected by obesity and ART. The effect of the ART combination on cellular studies, *ex vivo*, may yield interesting results in terms of underlying mechanisms. It would be interesting to elucidate the effect of this ART combination and in comparison to 2<sup>nd</sup> line ART as well in another model, such as an *in vitro* model.

Many of the mechanisms involved in the pathogenesis of ART-associated CVD disease need to be elucidated. For example, our study indicated that this ART combination may

contribute to insulin level dysregulation and hepatotoxicity, but this still needs to be described.

Drug administration in our study lasted 6 weeks. Long-term exposure to ART has been shown to elucidate more adverse effects. The long-term effects of this ART combination may in terms of obesity and a lean diet alone may generate interesting findings. Although our diet was high in sucrose and fat, ART may show other effects in terms of other diets.

The drug combination consisted only of three ART drugs combined. Numerous other ART drug combinations are available. A study comparing the effects of different drug combinations may indicate which combinations are safer to use. The individual effects of our three drugs on cardiovascular health may have also indicated to which extent each drug contributes to cardiovascular health and other associated adverse effects associated with this specific drug component.

In general, the cardiovascular effects of ART and HIV have only recently started to receive greater attention in the research world. With numerous drugs and drug combination available on the market, and limited research on the CV effects of these ARTs in human and animal studies available, leaves a great gap to fill in terms of scientific research.

## **5.4. Research Outputs Associated with This Study**

### Conference Contributions:

Year: 2015

Authors: F Everson, H Strijdom, T Ogundipe, T Grandjean and A Genis.

Reference: *Investigate the Cardiovascular Effects of Antiretroviral Therapy in Obesity. (Oral Presentation)*

Physiology Society of Southern Africa Annual Congress, Parys (Free State), 2015.

Year: 2015

Authors: A Genis, F Everson, T Ogundipe, T Grandjean and H Strijdom.

Reference: *NO, ROS and Pro-Inflammatory Signalling in Ischemia-Reperfused Hearts from Obese Rats Treated With 1st line ART's. (Poster Presentation)*

Physiology Society of Southern Africa Annual Congress, Parys (Free State), 2015.

Other outputs:

Year: 2014

Authors: F Everson, H Strijdom, T Ogundipe, T Grandjean and A Genis.

Reference: *Investigate the Cardiovascular Effects of Antiretroviral Therapy in Obesity.*

Annual Research Day, Faculty of Health Sciences, Stellenbosch University, 2014.

Year: 2015

Authors: F Everson, H Strijdom, T Ogundipe, T Grandjean and A Genis.

Reference: *The Effects of Antiretroviral Therapy on Cardiometabolic Parameters In a High Fat Diet Rat Model of Obesity.*

Annual Research Day, Faculty of Health Sciences, Stellenbosch University, 2015.

Year: 2015

Authors: F Everson, H Strijdom, T Ogundipe, T Grandjean and A Genis.

Reference: *Antiretroviral Therapy: The Cardiovascular Effects in a High Fat Diet Rat Model of Obesity.*

Annual Research Day, Department of Biomedical Sciences, Stellenbosch University, 2015.



## Appendix A - Preparation of HFD Food

The HFD food was prepared by soaking normal rat chow (600 g) in a 250 ml mixture of warm water and dissolved sucrose (140 g standard household sugar) for about 30 minutes. The soaked rat chow was thoroughly mixed with melted vegetable fat (4 X 125 g Holsom™), eight cans of full cream, sweetened, condensed milk (8 X 385 g / can; Clover™) and stored in a refrigerator for later use.

**Table A.1.** *Nutritional Value of Holsom™ Butter.*

	<b>Per 100 g</b>
Energy (kJ)	3700
Protein (g)	0
Glycaemic Carbohydrates (g)	0
Of which total sugar (g)	0
Total fat (g)	100
Of which saturated fat (g)	67.8
Trans fat (g)	<1
Monounsaturated fat (g)	26.4
Dietary fibre (g)	0
Total Sodium (mg)	0
Vitamin A (µg)	1380
Vitamin D (µg)	15

**Table A.2.** *Nutritional Values of Condensed Milk.*

	<b>Per 100 g</b>
Energy (kJ)	1381
Protein (g)	6.5
Glycaemic Carbohydrates (g)	54
Of which total sugar (g)	54
Total fat (g)	8.7
Of which saturated fat (g)	5.5
Dietary fibre (g)	0
Total Sodium (mg)	127
Calcium (mg)	284

## Appendix B - Method of Drug Dose Calculation

The daily dose (mg / kg) of each active ingredient for a human was calculated as follow:

$$AI_d = \frac{AI}{m_t}$$

$AI_d$  = Daily dose of active ingredient (mg / kg).  
 $AI$  = Active ingredient (mg).  
 $m_t$  = Average body mass of a human(kg).

(8.6 mg / kg / day EFV; 2.9 mg / kg / day FTC; 4.3 mg / kg / day TDF)

Daily dosage per animal (mg / kg) was calculated according to the USA FDA guidelines using a conversion factor of X 6 to convert human to rat ratio:

$$AI_{rat} = 6(AI_d)$$

$AI_{rat}$  = Daily dose of active ingredient for a rat (mg / kg)  
 $AI_d$  = Daily dose of active ingredient (mg / kg)

(EFV: 51.6 mg / kg / day; FTC: 17.4 mg / kg / day; TDF: 25.8 mg /kg / day).

Thus, for treated groups, the daily dose of active ingredients per animal needed was calculated weekly based on the average TBM of each group as follow:

$$AI_{d/r} = AI_{rat}(m_{avg})$$

$AI_{d/r}$  = Daily dose of active ingredient per rat (mg / kg).  
 $AI_{rat}$  = Active ingredient for a rat (mg).  
 $m_{avg}$  = Average TBM of rats in a group (kg).

The mass of the 3 active ingredients combined in each tablet was 1100 mg (600 mg EFV + 200 mg FTC + 300 mg TDF). The actual % (m / m) of the active ingredients in each tablet was calculated according to the mass of the tablet as follow:

$$AI_{\%} = \frac{AI_T}{m_T}$$

$AI_{\%}$  = % Mass active ingredient in the tablet.  
 $AI_T$  = Total mass of active ingredient, 1100 g.  
 $m_T$  = Total mass of the crushed tablet (g).

The amount (mg) of crushed powder of the tablet needed for a treated group with a certain average TBM (350 g per animal for example) was calculated as follow:

EFV:  $AI_{rat} = 51.6 \text{ mg / kg / day}$

$$AI_{d/r} = AI_{rat}(m_{avg})$$

$AI_{d/r}$  = Daily dose of active ingredient per rat (mg / kg).  
 $AI_{rat}$  = Active ingredient for a rat (mg).  
 $m_{avg}$  = Average TBM of rats in a group (kg).

= (51.6 mg / kg)(0.35 kg)  
 = 33.22 mg

If the % (m/m) of active ingredient is 68 % (for example), the amount (mg) of tablet powder weighed off was calculated as follow:

$$AI_w = AI_{d/r}(AI\%)$$

$$= 33.22 \text{ mg (68 \%)} \quad AI_w = \text{Mass of the tablet powder weighed off per rat (mg)}$$

$$= 22.58 \quad AI_{d/r} = \text{Daily dose of active ingredient per rat (mg / kg).}$$

$$AI\% = \text{Mass \% active ingredient in the tablet.}$$

$$m_p = \frac{m_T}{AI_w}$$

$$m_p = \text{Mass of the crushed tablet needed per rat daily (mg).}$$

$$m_T = \text{Total mass of the crushed tablet (mg).}$$

$$AI_w = \text{Mass of the tablet powder weighed off per rat (mg).}$$

To prepare enough of the FDC for 4 rats for 8 days:

$$M_{TP} = m_p(N)(d) \quad M_{TP} = \text{Total mass of crushed powder per group per week (g).}$$

$$m_p = \text{Mass of the crushed tablet needed per rat daily (mg).}$$

$$N = \text{Number of rats in the group.}$$

$$d = \text{Number of days}$$

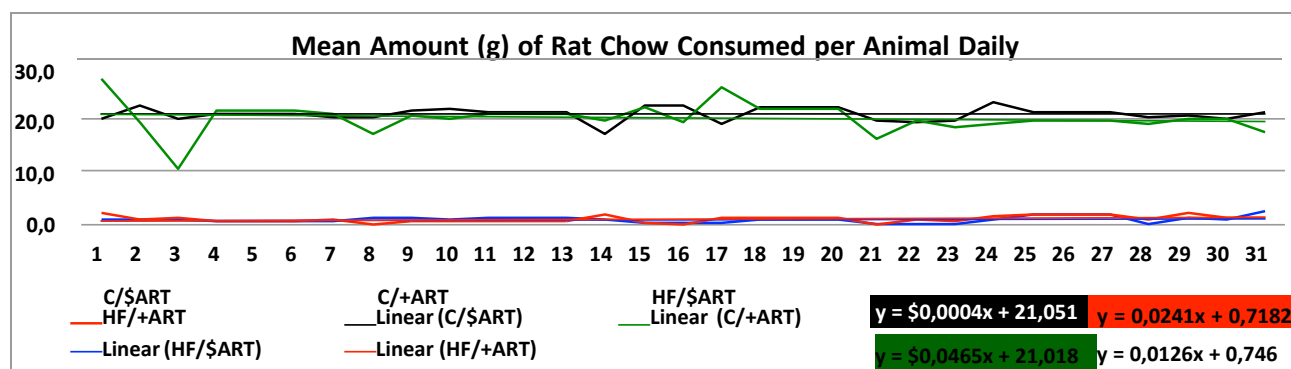
## Appendix C - Principles of the ELISA Assay

The kit is used for the non-radioactive quantification of insulin in mouse and rat sera. The assay is a sandwich ELISA based. The principal of the procedure include:

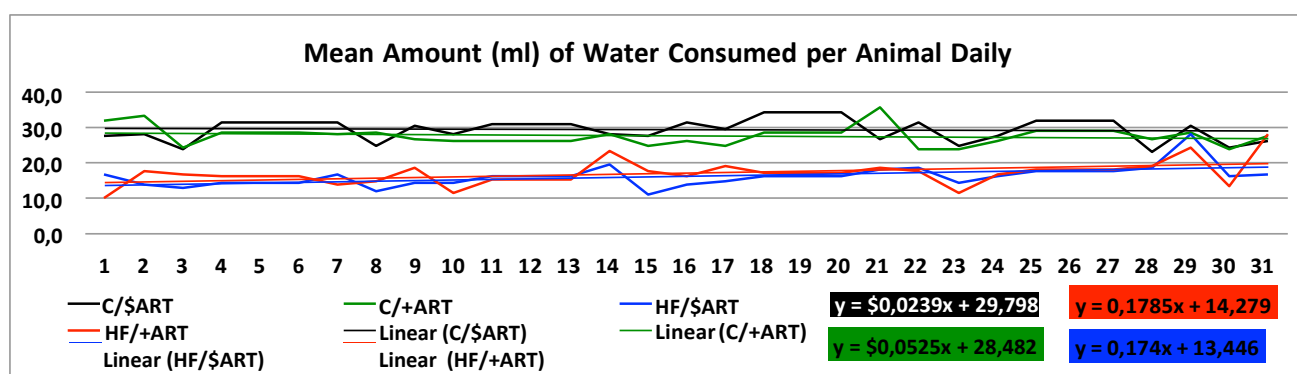
- a) Capturing of the insulin molecules from samples to the wells of a microtiter plate coated by pre-titered amount of a monoclonal mouse anti-rat insulin antibodies and the binding of biotinylated polyclonal antibodies to the captured insulin.
- b) The wash away of unbound materials from samples.
- c) The binding of horseradish peroxidase to the immobilised biotinylated antibodies.
- d) The wash away of free enzyme conjugates by monitoring horseradish peroxidase activities in the presence of the substrate 3,3',5,5'-tetramethylbenzidine.

The enzyme activity is measured spectrophotometrically by the increased absorbency at 450nm, corrected from the absorbency at 590nm, after acidification of formed products. Since the increase in absorbency is directly proportional to the amount of captured insulin in the unknown samples, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of rat insulin.

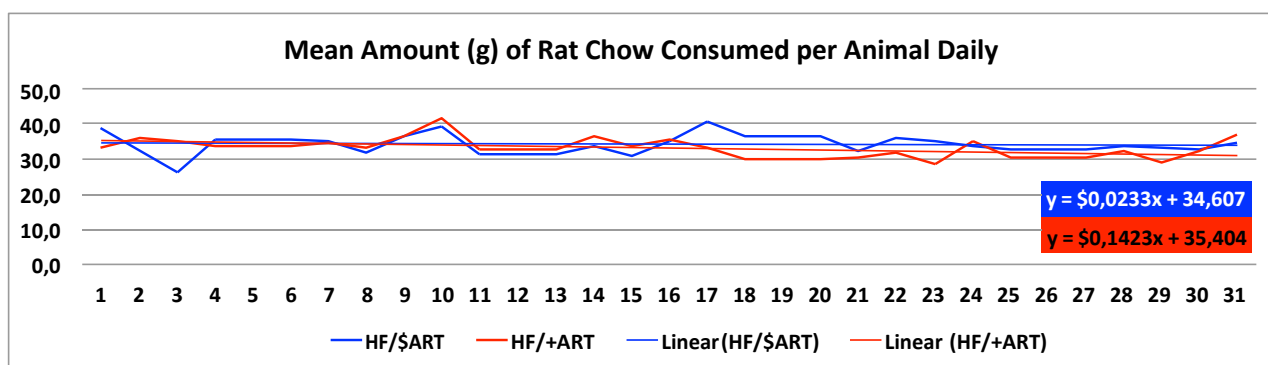
## Appendix D - Food and Water Monitoring



**Fig. D.1.** Mean Amount of Rat Chow Consumed / Animal (g / animal / day) for each Experimental Group for the First 31 Days During the Drug Treatment Programme with Linear Trend Lines. **Slope of Trend Line:** C/-ART: -0.00; HF/-ART: 0.05; C/+ART: -0.01; HF/+ART: 0.02. N = 7 - 8 / day / group.



**Fig. D.2.** The Mean Amount of Water Consumed / Animal (ml / animal / day) for the first 31 Days of the Drug Treatment Programme with Linear Trend lines. **Slope of Trend Line:** C/-ART: -0.00; HF/-ART: 0.17; C/+ART: -0.05; HF/+ART: 0.18. N = 7 - 8 / day / group.



**Fig. D.3.** The Mean Amount of HFD Food Consumed / Animal (g / animal / day) for the First 31 Days of the Drug Treatment Programme with Linear Trend Lines. **Slope of Trend Line:** HF/-ART: -0.02; HF/+ART: -0.14. N = 7-8 / day / group.

## Appendix E - Other Haemodynamic Data for Global Ischaemia-Reperfusion

**Table E.1.** Summary of Other Haemodynamic Data – Global Ischaemia-Reperfusion (data expressed as mean  $\pm$  SEM / group (% Recovery),  $p < 0.05$ ). **Abbreviations:** DP: Diastolic Pressure; MAP: Mean Arterial Pressure; PST: Peak Systolic Time; ET; Ejection Time; CT: Cycle Time; KP: Kinetic Power; PP: Pressure Power; dP/dTMax: Maximum Rate of Left Ventricular Pressure Rise; dP/dTMin: Maximum Rate of Left Ventricular Pressure Fall. **Pre-Ischaemia vs. Post-Ischaemia:** \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ ; \*\*\*\* =  $p < 0.0001$ .

Haemodynamic Data					
Pre\$Ischaemia vs. Post\$Ischaemia					
Mean $\pm$ SEM (% Recovery)		C/:ART (Pre: N = 16; Post: N = 6)	HF/:ART (Pre: N = 14; Post: N = 6)	C/+ART (Pre: N = 11; Post: N = 4)	HF/+ART (Pre: N = 13; Post: N = 6)
DP (mmHg)	Pre	84.63 $\pm$ 1.48	82.29 $\pm$ 1.381	84.27 $\pm$ 1.657	82.31 $\pm$ 1.402
	Post	71.67 $\pm$ 14.58	35.17 $\pm$ 16.57 ****	60.75 $\pm$ 0.35 (72.09%)	40.33 $\pm$ 18.07 (49.0%) ***
% Recovery		84.69 %	42.73 %	72.09 %	49.00 %
MAP (mmHg)	Pre	98.43 $\pm$ 1.225	95.12 $\pm$ 1.581	95.51 $\pm$ 1.55	93.69 $\pm$ 1.727
	Post	79.12 $\pm$ 15.95	38.60 $\pm$ 18.31 ****	68.00 $\pm$ 1.73	45.2 $\pm$ 20.26 (48.24%) ***
% Recovery		80.38 %	40.58 %	71.20 %	48.24 %
ET (sec)	Pre	0.078 $\pm$ 0.00	0.075 $\pm$ 0.002	0.071 $\pm$ 0.00	0.08 $\pm$ 0.00
	Post	0.058 $\pm$ 0.01 (75.26%)	0.03 $\pm$ 0.02 ****	0.064 $\pm$ 0.00	0.05 $\pm$ 0.01 **
% Recovery		75.26 %	34.1 %	89.80 %	62.74 %
CT (sec)	Pre	0.29 $\pm$ 0.022	0.26 $\pm$ 0.01	0.24 $\pm$ 0.01	0.25 $\pm$ 0.01
	Post	0.20 $\pm$ 0.04	0.08 $\pm$ 0.05 ****	0.33 $\pm$ 0.04	0.02 $\pm$ 0.05
% Recovery		71.46 %	31.00 %	130.31 %	81.89 %
PSP (mmHG)	Pre	105.10 $\pm$ 1.57	100.80 $\pm$ 2.05	101.70 $\pm$ 1.69	99.15 $\pm$ 2.03
	Post	83.32 $\pm$ 16.79	41.00 $\pm$ 19.17 ****	86.0 $\pm$ 8.37	66.83 $\pm$ 16.79 *
% Recovery		77.80 %	40.94 %	82.94 %	64.44 %

## Appendix F - Haemodynamic Data for Regional Ischaemia-Reperfusion

**Table F.1.** Summary of Haemodynamic Data – Regional Ischaemia-Reperfusion (data expressed as mean  $\pm$  SEM / group (% Recovery),  $p < 0.05$ ). **Abbreviations:** HR: Heart Rate; AO: Aortic Output; CF: Coronary Flow; CO: Total Cardiac Output; PSP: Peak Systolic Pressure; Wt: Total Power; Pre: Pre-ischaemia-reperfusion; Post: Post-Ischaemia-reperfusion. **Pre-Ischaemia vs. Post-Ischaemia:** \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ ; \*\*\*\* =  $p < 0.0001$ .

Haemodynamic Data					
Pre\$Ischaemia vs. Post\$Ischaemia					
Mean $\pm$ SEM (% Recovery)		C/:ART (Pre: N = 16; Post: N = 8)	HF/:ART (Pre: N = 14; Post: N = 7)	C/+ART (Pre: N = 11; Post: N = 4)	HF/+ART (Pre: N = 13; Post: N = 8)
HR (BPM)	Pre	224.80 $\pm$ 12.67	234.80 $\pm$ 8.72	245.90 $\pm$ 6.18	259.40 $\pm$ 17.47
	Post	162.70 $\pm$ 37.66	227.30 $\pm$ 17.07	230.20 $\pm$ 13.97	229.60 $\pm$ 57.86
% Recovery		72.38 %	96.80 %	93.61 %	88.51 %
AO (ml / min)	Pre	42.09 $\pm$ 1.51	41.21 $\pm$ 1.48	42.36 $\pm$ 1.87	38.46 $\pm$ 1.57
	Post	16.58 $\pm$ 5.34 ****	7.08 $\pm$ 5.27 ****	14.50 $\pm$ 5.85 ****	7.167 $\pm$ 4.83 ****
% Recovery		14.73 %	7.28 %	22.24 %	15.97 %
CF (ml / min)	Pre	14.13 $\pm$ 0.7353	14.11 $\pm$ 0.5114	15.36 $\pm$ 0.74	16.0 $\pm$ 1.13
	Post	8.40 $\pm$ 1.90 ***	9.75 $\pm$ 0.94 *	15.42 $\pm$ 0.99	12.36 $\pm$ 1.81
% Recovery		59.45 %	69.10 %	100.39 %	76.87 %
CO (ml / min)	Pre	56.22 $\pm$ 1.84	55.32 $\pm$ 1.73	57.91 $\pm$ 1.81	54.54 $\pm$ 2.41
	Post	14.60 $\pm$ 4.59 ****	12.75 $\pm$ 3.028 ****	26.25 $\pm$ 2.813 ***	18.50 $\pm$ 4.39 ****
% Recovery		25.97 %	23.05 %	45.33 %	33.92 %
PST (sec)	Pre	0.07 $\pm$ 0.00	0.07 $\pm$ 0.00	0.06 $\pm$ 0.01	0.07 $\pm$ 0.00
	Post	0.05 $\pm$ 0.01	0.10 $\pm$ 0.02 *	0.08 $\pm$ 0.02	0.07 $\pm$ 0.02
% Recovery		70.07 %	157.24 %	131.95 %	104.42 %
Wt (mW)	Pre	13.24 $\pm$ 0.52	12.35 $\pm$ 0.43	13.24 $\pm$ 0.53	12.24 $\pm$ 0.61
	Post	2.91 $\pm$ 0.99 ****	2.14 $\pm$ 0.79 ****	4.82 $\pm$ 0.49 ****	3.47 $\pm$ 1.02 ****
% Recovery		21.99 %	17.30 %	36.37 %	29.34 %
KP (mW)	Pre	0.13 $\pm$ 0.03	0.09 $\pm$ 0.01	0.11 $\pm$ 0.00	0.08 $\pm$ 0.01
	Post	0.02 $\pm$ 0.01	0.01 $\pm$ 0.01 ***	0.04 $\pm$ 0.02	0.01 $\pm$ 0.01
% Recovery		16.64 %	8.20 %	41.71 %	18.03 %
PP (mW)	Pre	13.10 $\pm$ 0.50	12.26 $\pm$ 0.42	13.14 $\pm$ 0.52	12.08 $\pm$ 0.51
	Post	6.05 $\pm$ 1.57 ****	2.43 $\pm$ 1.68 ****	6.32 $\pm$ 2.25 ***	3.56 $\pm$ 1.41 ****
% Recovery		46.21 %	18.32 %	48.12 %	29.43%
dP/dTMax	Pre	18.17 $\pm$ 1.71	17.30 $\pm$ 0.91	21.33 $\pm$ 1.68	15.24 $\pm$ 1.19
	Post	10.58 $\pm$ 2.260 *	4.42 $\pm$ 2.91 ****	11.58 $\pm$ 2.14 *	9.38 $\pm$ 2.56
% Recovery		58.23 %	25.53 %	54.29 %	61.52 %
dP/dTMin	Pre	916.65 $\pm$ 1.20	917.08 $\pm$ 1.10	917.62 $\pm$ 1.32	916.36 $\pm$ 1.36
	Post	910.01 $\pm$ 2.29 *	93.75 $\pm$ 2.49 ****	98.83 $\pm$ 2.00 *	97.19 $\pm$ 2.23 **
% Recovery		60.12 %	21.96 %	50.13 %	43.97 %



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